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PRINCIPAL INVESTIGATOR: Hong Sun, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

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13. ABSTRACT *(Maximum 200 words)*

PTEN is an important tumor suppressor that is commonly mutated in a wide variety of human cancers. Recent studies have suggested that the PTEN protein can in vitro dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PIP3), a product of PI 3-kinase. We have generated mouse embryonic stem (ES) cells in which either one or both copies of the *PTEN* genes were deleted. Using the isogenic PTEN+/+ and PTEN-/- cells, we showed that PTEN deficiency leads to an increase of PIP3 levels and enhanced activation of Akt, indicating that PTEN indeed negatively regulates the PI 3-kinase/Akt signaling pathway *in vivo*. Characterization of PTEN+/+ and PTEN-/- ES cells further demonstrated that PTEN regulates two critical cellular processes: cell cycle progression and apoptosis. PTEN-/- ES cells exhibit an advanced S-phase entry and the marked decrease in levels of p27KIP1, an inhibitor for cyclin-dependent kinases. We have further shown that expression of PTEN in PTEN-deficient human glioblastoma cells causes G1 cell cycle arrest and accumulation of p27KIP1. Our studies suggest that PTEN in vivo regulates PI 3-kinase signaling pathway and a critical target for this pathway is p27KIP1. p27KIP1 is a well known prognosis marker for human breast cancer.

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1. Li, D-M. and Sun, H. (1998) PTEN/MMAC1/TEP1 suppresses tumorigenecity and blocks cell cycle progression in human glioblastoma cells. *Proc. Natl. Acad. Sci. USA* 95, 15406-15411.
2. Sun, H., Lesche, R., Li, D-M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999) PTEN modulates cell cycle progression and cell survival by regulating PIP3 and Akt/PKB signaling pathway. *Proc. Natl. Acad. Sci. USA* 96, 6199-6204.

# INTRODUCTION

We have previously cloned a novel protein, TEP1, based on its sequence homology to members in the dual specificity subfamily of protein tyrosine phosphatases [1]. The gene encoding TEP1 maps to chromosome 10q23, a locus frequently deleted in human glioblastomas and prostate cancers. The TEP1 gene was found to be identical to PTEN or MMAC1, a tumor suppressor gene independently isolated by positional cloning method [2,3]. Accumulating evidence indicates that TEP1/PTEN/MMAC1 (hereafter referred to as PTEN) is encoded by a major tumor suppressor gene which is frequently mutated or deleted in a wide variety of human cancers including several familial, cancer predisposition disorders such as Cowden disease and Bannayan-Ruvalcaba-Riley syndrome [4]. Our group was the first to demonstrate that in vitro PTEN protein possesses an intrinsic phosphatase activity towards phosphotyrosine containing proteins [1]. It was later shown that PTEN could also dephosphorylate, in vitro, phosphatidylinositol (3,4,5) trisphosphate, a product of phosphatidylinositol 3-kinase (PI 3-kinase) [5]. Many tumor derived mutations have been mapped into the conserved motif of the phosphatase domain on PTEN, suggesting that the phosphatase activity is critical for PTEN tumor suppression function. More recently, inactivation of PTEN in a mouse model has confirmed the role of PTEN as a bona fide tumor suppressor [6].

In my original proposal, I have outlined strategies to investigate the involvement of PTEN in human breast cancers. In order to clearly and definitely define the contribution of PTEN deficiency to human breast cancers, we need to understand the cellular processes and the signaling pathways regulated by PTEN. We have made great progresses towards these goals in our last year's research, which was supported by my breast cancer grant (DAMD17-98-1-8271) and my Pew Scholar Award. We have demonstrated that PTEN regulates cell cycle progression at the G1/S transition in both human tumor cells and mouse Pten knock-out cells [7,8]. Our observation of PTEN regulating cell cycle progression provides a molecular explanation for the marked over-proliferation and increase of S-phase cell population phenotype seen in Pten-/- mouse embryos [9]. Second, we have identified $p27^{KIP1}$, an inhibitor of G1 cyclin-dependent kinases, as a specific downstream target for the PTEN-regulated signaling pathway [7,8]. Emerging evidence indicates that p27 serves as a critical cell cycle regulator that mediates the growth factor-dependent cell cycle progression and entry of the S-phase. The level of p27 is also a good prognosis marker for human breast cancer. Our studies raise an interesting possibility that PTEN deficiency or haploinsufficiency may lead to down-regulation of p27 which in turn promotes breast cancer progression. Third, we have employed mouse Pten-gene knockout cells to show that PTEN in vivo regulates phosphatidylinositol 3-kinase (PI 3-kinase) and Akt/PKB signaling pathway [8]. PI 3-kinase pathway has long been implicated in tumor invasion and metastasis. We have established methods to effectively express PTEN in human tumor cells which will be used to further characterize the roles of PTEN in regulation of breast cancer cell migration and invasion.

# BODY

## Aim #1. To investigate the occurrence of mutations in the PTEN gene in sporadic breast cancer.

In my original proposal, I have proposed to investigate the occurrence of mutations in the PTEN gene in sporadic breast cancers. At the time when this proposal was funded, there were reports that PTEN gene mutations were found at low frequency in human breast cancer [10]. However, the analysis methods used may fail to detect cases in which only one of the two copies of the PTEN gene is mutated. To circumvent such problems, we want to establish assays for the PTEN functional status, which in turn calls upon the identification of the cellular processes regulated by PTEN. We therefore modified this aim to examine whether PTEN deficiency or PTEN haploinsufficiency will affect cell proliferation, differentiation or apoptosis.

To create an isogenic pair of cells that differ only in PTEN, we introduced a null mutation into the mouse *Pten* gene by homologous recombination in mouse embryonic stem (ES) cells. So far we have successfully generated six independent Pten+/- ES cell lines. To facilitate the study of PTEN function at the cellular level, we generated Pten-/- ES cells by selecting with a higher dose of G418. To avoid potential clonal variations, each of the following experiments was repeated and confirmed by using several independent ES clones. The Pten-/- ES cells appeared to reach high cell density faster. To determine whether Pten-/- cells have altered growth properties, we performed a growth competition experiment. Equal numbers of Pten+/+ cells were mixed with Pten+/- or Pten-/- cells and co-cultured. Cells were passed every three days to avoid growth saturation. At the end of the third passage (corresponding to 13-14 doublings of normal ES cells), the Pten-/- ES cells had out-grown the Pten+/+ cells, as revealed by diagnostic restriction DNA fragments using Southern blot. As a control, when Pten+/+ and Pten+/- cells were co-cultured, no competition of growth was observed. These results suggest that deletion of the *Pten* gene provides cells with a growth advantage, possibly by shortening the cell cycle time (see[8] for detail).

To determine whether the PTEN deficiency promotes cell cycle progression, we synchronized Pten+/+ and Pten-/- ES cells to compare their cell cycle time. Since ES cells do not exhibit contact inhibition and respond poorly to serum deprivation, we used the mitotic shake-off method. Mitotic cells were collected after colcemid (4 hrs) treatment and released into fresh medium. S-phase entry was monitored by incorporation of [$^3$H]-thymidine. [$^3$H]-thymidine incorporation occurred about three hours after release from the mitotic block (Fig. 1A), suggesting that ES cells have a G1 phase of about 3-4 hours. Pten-/- cells reproducibly showed an earlier (approximately 0.5 -1 hr advance) entry into S-phase compared to Pten+/+ cells. Since the synchronized ES cells have a cell cycle time of approximately 9-10 hours, the advanced S phase entry we observed in Pten-/- cells shortened the cell cycle time by 5-10%.

To determine the cause of early S-phase entry in Pten-/- cells, we examined the levels of G1 cell cycle regulators, including two major G1 CDK inhibitors, p21 and p27. Consistent with previous reports, p21 was not detectable in ES cells. However, p27 was readily detected in ES cells. In asynchronously growing cells, there was a marked reduction of p27 level (about 5-fold) in Pten-/- cells as compared with that of Pten+/+ cells (Fig. 1B). In contrast, the levels of other G1 cell cycle regulators, such as cyclin D1, cyclin E, cyclin A, CDK2, showed no detectable alteration (Fig. 1B). These results suggest that p27 is a selective target regulated by PTEN [8].

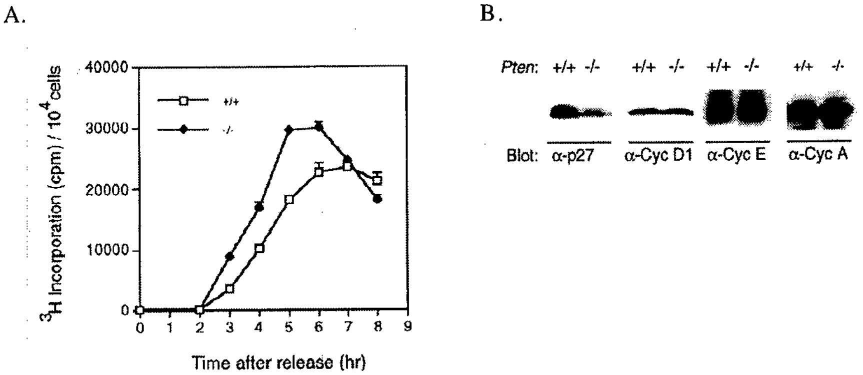


Fig. 1. (A) [$^3$H]-thymidine incorporation. Cells were synchronized by mitotic shake-off. At each time point, cells ($2x10^5$) were pulse-labeled with [$^3$H]-thymidine for 1 hr and [$^3$H]-thymidine incorporation was measured. (B) Cell lysates (40 µg) from actively growing cells were either directly blotted with p27 or cyclin D1 antibody. 10 fold more cell lysates (400 µg) were used for IP/Western blot with cyclin E or cyclin A antibody.

## Aim #2. To examine the expression of the PTEN gene in breast cancer tumor samples

In my original proposal, I have proposed to study the expression of PTEN gene in breast cancer tumor samples. To more efficiently detect the functional status of PTEN in breast cancer tumor samples, we started to identify the cell signaling pathway regulated by PTEN. Our studies have shown that PTEN regulates PI 3-kinase/Akt signaling pathway in vivo. We should now therefore be able to determine the functional status of PTEN in these tumor samples by monitor the activities of Akt.

PI 3-kinase plays a major role for cells to progress from G1 to S phase. PI 3-kinase regulates the production of PIP3, which acts as a second messenger of the PI 3-kinase signaling pathway. It was recently reported that PTEN could dephosphorylate PIP3 *in vitro* [5]. The *Pten* knock-out cells provided us a unique opportunity and a clean genetic system to test whether PTEN functions *in vivo* in the PI 3-kinase signaling pathway. Using the genetically defined Pten-/- and Pten+/+ but otherwise isogenic ES cells, we tested the levels of PIP3 in cells that had been stimulated with IGF-1. Cells were metabolically labeled and phospholipids were extracted and analyzed by an established thin layer chromatography (TLC) to separate PIP3 away from other forms of phosphoinositides and phospholipids. As shown in Fig. 2, deletion of PTEN caused elevation of the PIP3 level both in the magnitude and duration following IGF-1 stimulation. In contrast, the levels of PIP and PIP2 were largely unaffected by PTEN deficiency. The basal level (without IGF-1 stimulation) of PIP3 is comparable in Pten+/+ and Pten-/- cells. This increase in the PIP3 level in Pten-/- ES cells is quite reproducible and is comparable to the elevated PIP3 level induced by the over-expression of a constitutively active form of PI 3-kinase (form p110*), as reported previously. These observations suggest that PIP3 is target of PTEN in vivo [8].

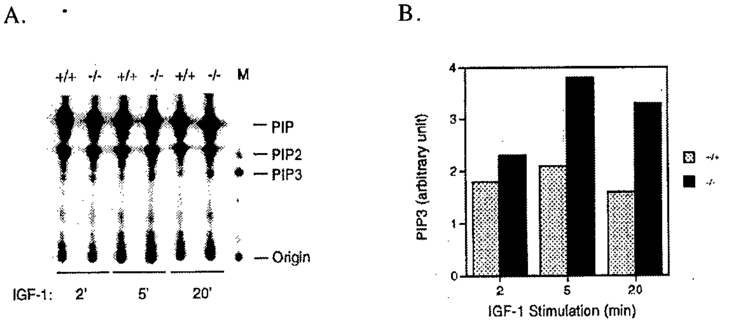


Fig. 2. (A) PIP3 levels in *Pten+/+* and *Pten-/-* ES cells. Cells were starved in a serum-free medium for 16 hours, labeled with [$^{32}$P]-orthophosphate for 2 hours, and stimulated by IGF-1. Phospholipids were extracted and analyzed on a TLC plate. Assignment of PIP, PIP2 and PIP3 was done according to in vitro $^{32}$P-labeled PIP, PIP2, or PIP3 standards, which were prepared using PI 3-kinase and appropriate lipid substrates and chromatographed in parallel. In lane M, [$^{32}$P]-labeled PIP3 is shown as a marker. (B) Quantitation of PIP3 levels in *Pten+/+* and *Pten-/-* ES cells. The amount of radioactivity (in cpm) corresponding to PIP3 was measured with PhosphoImager and presented as arbitrary unit.

We have further examined the phosphorylation and activation status of Akt/PKB, a downstream signaling kinase known to be activated by phosphoinositide PIP3 signaling. The sustained high level of Akt phosphorylation in Pten-/- cells suggests that the increase in PIP3 accumulation caused by PTEN deficiency is sufficient to induce the activation of Akt (Fig 3A). The level of phosphorylated Akt showed marked sensitivity to the *Pten* gene dosage and no changes in the protein level of Akt could be detected. To determine whether PTEN deficiency affects signaling pathways other than the Akt pathway, we examined the phosphorylation status of MAP kinases and focal adhesion kinase (FAK). Our experiment revealed that the phosphorylation of MAP kinases and FAK were not affected by the PTEN deletion (Fig. 3B). In addition, the phosphorylation status of p85, the regulatory subunit of p85/p110 PI 3-kinase was also not affected by the PTEN. These studies suggest that PTEN deficiency leads to selective activation of PIP3/Akt signaling pathway [8].

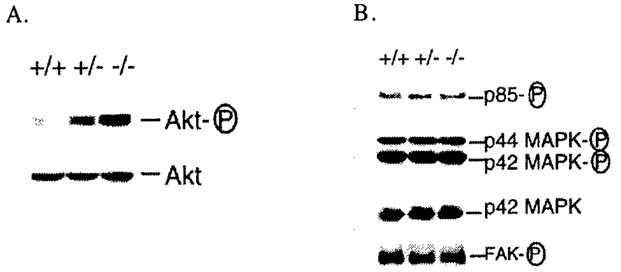
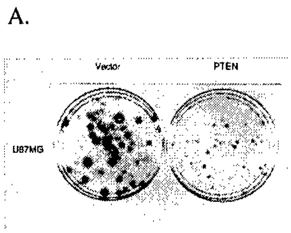


Fig. 3. (A) Phosphorylation status of Akt in actively growing ES cells. Cell lysates from log-phase growing *Pten+/+*, *Pten+/-* or *Pten-/-* ES cells were analyzed with antibodies against phospho-Akt (serine 473) or total Akt, respectively. (B) Phosphorylation status of PI 3-kinase, MAP kinases, and FAK in actively growing cells. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody 4G10 and blotted with p85 or FAK antibodies, respectively. Alternatively, cell lysates were examined by Western blot analysis with antibody against phospho-MAPKs or p42 MAPK, respectively.

## Aim #3. To test if PTEN functions as a suppressor for tumor cell invasion and metastasis

To examine how PTEN function as a tumor suppressor, we first examined the effect of PTEN expression in human glioblastoma U87MG cells. We initially chose U87MG cells since it has been reported to be deficient for PTEN [3]. We have found that PTEN exerts potent growth suppression in U87MG cells (Fig. 4A) but not in other tumor cells such as colon carcinoma cells DLD1 cells, suggest that PTEN growth suppression function may be highly cell type specific [7]. We have therefore used U7MG cells to dissect the mechanism of PTEN-mediated growth suppression.



B. Cell cycle profile.

| Retrovirus | G0/G1 | S | G2/M |
|---|---|---|---|
| Vector | 55 | 28 | 17 |
| PTEN | 71 | 12 | 17 |
| PTEN(CS) | 54 | 28 | 18 |

Fig. 4. (A) U87MG cells were infected with either empty-vector or PTEN-expressing retroviruses. The infected cells were selected in puromycin containing medium (4 weeks), fixed, and stained with Giemsa dye. (B) U87-EcoR cells were infected with retroviruses carrying either empty vector, or expressing PTEN or PTEN(CS). 48 hours after infection, cells were pulse-labeled with BrdU and analyzed for cell cycle profile by fluorescence-activated cell sorting method.

To improve the retrovirus delivery efficiency into human cells, we have established U87MG derivatives (U87-EcoR) that stably express a murine ecotropic receptor. Expression of PTEN in U87-EcoR cells led to more than 50% reduction in the S phase cell population and a substantial increase in the G0/G1 cells (Fig. 4B). Such an arrest was also depended on its phosphatase activity, as very little effect was observed when the catalytically-inactive PTEN(CS) mutant was expressed (Fig. 4B). To determine the mechanism of cell cycle arrest, we examined the levels of CDK inhibitors $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ in cells infected with retroviruses either containing the empty vector, PTEN, or PTEN(CS). Expression of PTEN but not PTEN(CS) led to a significant elevation of p27 (Fig. 5A). No changes were found in the levels of p21, cyclin D, or CDK2 (Fig. 5A). These results are in complement to our studies in mouse Pten-/- cells, suggesting that PTEN play a role in regulation of cell cycle progression and p27KIP1 is a critical downstream target PTEN-regulated pathway [7,8].

To investigate whether inhibition of PI 3-kinase/Akt pathway is responsible for PTEN-mediated growth suppression, we examined the status of Akt in cells infected with retroviruses carrying PTEN, PTEN(CS), or the vector. Substantial reduction in the amount of the Ser473 phosphorylated, active forms of Akt were observed in cells expressing PTEN, while the total protein level of Akt was not affected (Fig. 5B). In contrast, the active and phosphorylated forms of both p42 and p44 MAP kinases were not detectably altered by PTEN, as detected by an antibody that recognizes the Thr202/Tyr204 phosphorylated p42 and p44 MAP kinases (Fig. 5B). The level of p42 MAP kinase was also not affected by PTEN expression.

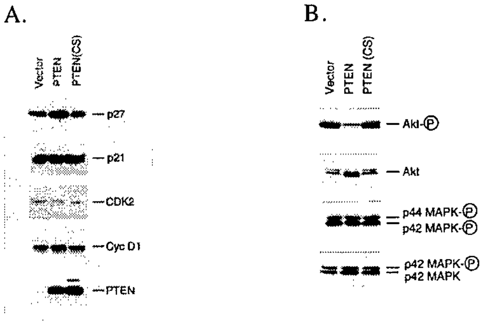


Fig. 5. (A) Western blot analysis. U87-EcoR cells were infected with indicated retroviruses and cell lysates were analyzed by Western blotting with p27, p21, CDK2, cyclin D1 or PTEN antibodies, respectively. (B) Phosphorylation status of Akt and MAPKs. U87-EcoR cells were infected with indicated retroviruses. Cell lysates were blotted with antibodies against phospho-Akt/PKB, phospho-MAPKs,or with regular anti-Akt or anti-p42 MAPK antibodies, respectively.

We are currently extending our studies to human breast cancer cells. We are in the process of establishing derivatives of MDA-MB-231 and MDA-MB-436 cell lines which express the murine ecotropic receptors. Such derivatives should allow us to efficiently express PTEN in the whole population of these human breast cancer cells and assay the effect of PTEN expression on cell migration and invasion.

## KEY RESEARCH ACCOMPLISHMENTS

Our stuides in the past year have demonstrated that PTEN in vivo functions as a phosphatase for phosphatidylinoside (3,4,5)-trisphosphate. We have shown that in mammalian systems, PTEN modulates cell cycle progression and apoptosis. Our findings are summarized as below:

1. Demonstrate that the in vivo signaling pathway regulated by PTEN is PI 3-kinase/Akt pathway.

2. Demonstrate that cell cycle progression and apoptosis are critical cellular processes regulated by PTEN.

3. Identify $p27^{KIP1}$, a CDK inhibitor, as a specific target for PTEN-regulated signaling pathway.

## REPORTABLE OUTCOMES

### Manuscripts

1. Li, D-M. and Sun, H. (1998) PTEN/MMAC1/TEP1 suppresses tumorigenecity and blocks cell cycle progression in human glioblastoma cells. *Proc. Natl. Acad. Sci. USA* 95, 15406-15411.

2. Sun, H., Lesche, R., Li, D-M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999) PTEN modulates cell cycle progression and cell survival by regulating PIP3 and Akt/PKB signaling pathway. *Proc. Natl. Acad. Sci. USA* 96, 6199-6204.

### Abstracts

1. Da-Ming Li, Yu Yuan and Hong Sun (1998) Studies on the novel members in the dual-specificity phosphatase subfamily of protein tyrosine phosphatases that are involved in cell growth control. *FESEB Conference on Phosphatases*, Copper Mountain, Colorado.

2. Da-Ming Li, Yu Yuan and Hong Sun (1998) TEP1/PTEN/MMAC1 Phosphatase Suppresses the Tumorigenicity and Cell Cycle Progression of Human Glioblastoma Cells. *Cancer Genetics & Tumor Suppressor Genes*, Cold Spring Habor Laboratory, New York.

3. Ralf Lesche, Da-Ming Li, Hong Sun and Hong Wu (1998) Functional analysis of the candidate tumor suppressor gene PTEN by gene targeting. *Cancer Genetics & Tumor Suppressor Genes*, Cold Spring Habor Laboratory, New York.

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Principal Investigator (Hong Sun)
PTEN Tumor Suppressor and Cell Cycle Regulation
National Institute of Health/National Cancer Institute - RO1CA77695
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## CONCLUSIONS

Our studies in the past year have demonstrated that the in vivo substrate for the PTEN tumor suppressor is phosphatidylinoside (3,4,5)-trisphosphate. Identification of the in vivo signaling pathway for PTEN should help to design more sensitive assay to diagnose loss of PTEN function in breast tumor samples. We have further shown that PTEN modulates cell cycle progression and a critical target for PTEN-regulated pathway is the CDK inhibitor p27KIP1. p27KIP1 is known as an important prognosis marker for human breast cancer. In our future studies, we will examine how loss of PTEN contributes to breast cancer progression, which is likely mediated through increased cell migration and invasion of PTEN-deficient cells.

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Cell Biology

# PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway

HONG SUN*†, RALF LESCHE‡§, DA-MING LI*§, JOANNA LILIENTAL‡¶, HUI ZHANG*, JING GAO‡, NADIA GAVRILOVA*, BRENDA MUELLER‖, XIN LIU‖, AND HONG WU†‡

*Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520; and ‡Howard Hughes Medical Institute and Department of Molecular and Medical Pharmacology, and Departments of ¶Microbiology and Molecular Immunology, and ‖Pathology and Laboratory Medicine, University of California, 650 Circle Drive South, Los Angeles, CA 90095-1735

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**ABSTRACT** **To investigate the molecular basis of PTEN-mediated tumor suppression, we introduced a null mutation into the mouse *Pten* gene by homologous recombination in embryonic stem (ES) cells. *Pten*$^{-/-}$ ES cells exhibited an increased growth rate and proliferated even in the absence of serum. ES cells lacking PTEN function also displayed advanced entry into S phase. This accelerated $G_1$/S transition was accompanied by down-regulation of p27$^{KIP1}$, a major inhibitor for $G_1$ cyclin-dependent kinases. Inactivation of PTEN in ES cells and in embryonic fibroblasts resulted in elevated levels of phosphatidylinositol 3,4,5,-trisphosphate, a product of phosphatidylinositol 3 kinase. Consequently, PTEN deficiency led to dosage-dependent increases in phosphorylation and activation of Akt/protein kinase B, a well-characterized target of the phosphatidylinositol 3 kinase signaling pathway. Akt activation increased Bad phosphorylation and promoted *Pten*$^{-/-}$ cell survival. Our studies suggest that PTEN regulates the phosphatidylinositol 3,4,5,-trisphosphate and Akt signaling pathway and consequently modulates two critical cellular processes: cell cycle progression and cell survival.**

The tumor susceptibility gene encoding PTEN/MMAC1/TEP1 (1–3) is mutated at high frequency in many primary human cancers and several familial cancer predisposition disorders (4). PTEN contains the sequence motif that is highly conserved in the members of the protein tyrosine phosphatase family. PTEN has been shown *in vitro* to possess phosphatase activity on phosphotyrosyl, phosphothreonyl-containing substrates (3, 5) and more recently, on phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of phosphatidylinositol 3 (PI3) kinase (6). Many cancer-related mutations have been mapped within the conserved catalytic domain of PTEN, suggesting that the phosphatase activity of PTEN is required for tumor suppressor function. In addition, wild-type PTEN, but not mutant derivatives lacking phosphatase activity, suppresses the growth of glioblastoma cells and their tumorigenecity in nude mice (7–9), confirming the functional relevance of the PTEN phosphatase domain for tumor suppression. Very recently, inactivation of PTEN in a mouse model has confirmed the role of PTEN as a bona fide tumor suppressor (10). However, the exact function of PTEN in regulation of cell growth and tumorigenesis remains unclear.

In this study, we have investigated the molecular basis underlying the tumor suppression function of PTEN by using a combination of molecular genetic, cell biological, and biochemical approaches. We have identified PIP3, a product of PI3 kinase, as an intracellular target of PTEN. Our studies suggest that PTEN acts as a negative regulator for the PI3-kinase/Akt signaling pathway, which controls and coordinates two major cellular processes: cell cycle progression and cell death.

## MATERIALS AND METHODS

**Generation of *Pten*$^{-/-}$ Embryonic Stem (ES) and Mouse Embryonic Fibroblasts (MEF) Cell Lines.** Genomic DNA clones corresponding to the *Pten* locus were isolated from an isogenic 129(J1) genomic library (11). The targeting vector, pKO-1, contains the PGKneopA cassette flanked by 8.0-kb *Kpn*I–*Apa*I fragment (5′ arm) and 3-kb *Bam*HI–*Xba*I fragment (3′ arm) in the backbone of pBluescript vector. Linearized pKO-1 plasmid (25 μg) was electroporated into $1 \times 10^7$ J1 ES cells as described (11). G418$^r$ ES clones were isolated and expanded. Genomic DNAs were prepared for Southern blot analysis. To obtain ES clones homozygous for the *Pten* deletion, heterozygous ES clones were subjected to a higher G418 selection (5 mg/ml). Homozygous deletion was confirmed by Southern blot analysis. ES cell clones homozygous for the *Pten* deletion were injected into BALB/c blastocysts. Chimeric embryos were collected at embryonic day 14–15 gestation. MEF cells were prepared and subjected to G418 (400 μg/ml) selection for 10 days to eliminate wild-type (WT) cells. *Pten* deletion then was further confirmed by Southern and Western blot analyses.

**Colcemid Block and Mitotic Shake-Off.** ES cells were passaged twice without feeder support to remove *Pten*$^{+/+}$ feeder cells. Mitotic cells were prepared according to Savatier *et al.* (12). An equal number of mitotic cells were seeded on gelatinized plates and incubated to allow synchronized cell cycle reentry. Cytospin of collected cells followed by Wright staining showed that this procedure yielded 90–95% mitotic cells (data not shown).

**Antibodies, Western Blot Analysis, and Kinase Assay.** Cell lysate preparation, immunoprecipitation, Western blot analysis, and histone H1 kinase assay were carried out as described (9). Antibodies specific for p27$^{KIP1}$ (sc-528), cyclin D1 (R-124), p21$^{CIP1/WAF1}$ (sc-397), and mouse cyclin E (sc-481) were obtained from Santa Cruz Biotechnology. Antibodies specific for cyclin A, cyclin-dependent kinase (CDK2) (13), and PTEN (9) have been described. Antibodies for Bad (B36420) and focal adhesion kinase (FAK) (F15020) were from Transduction Laboratories, Lexington, KY. Antiphosphotyrosine antibody 4G10 and anti-

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Abbreviations: ES, embryonic stem; PIP, phosphatidylinositol 4-phosphate; PI3, phosphatidylinositol 3; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5,-trisphosphate; MEF, mouse embryonic fibroblasts; WT, wild type; CDK, cyclin-dependent kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; IGF-I, insulin-like growth factor I; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; FAK, focal adhesion kinase.

[†] To whom reprint requests should be addressed. e-mail: hong.sun@yale.edu or hwu@mednet.ucla.edu.

[§] R.L. and D.-M.L. contributed equally to this work.

p85 subunit of PI3 kinase antibody (06–195) were obtained from Upstate Biotechnology. Anti-Akt/protein kinase B (PKB) and anti-mitogen-activated protein kinase (MAPK) antibodies were from New England Biolabs.

**Phospholipids Analysis.** Phospholipid extraction and TLC analysis were performed according to Trynor-Kaplan *et al.* (14). To prepare $^{32}$P-labeled molecular weight markers for phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and PIP3, PI3 kinase was immunoprecipitated from insulin-like growth factor (IGF-I)-stimulated 293 cells with anti-p85 antibody and used to phosphorylate the lipid substrates phosphatidylinositol (Sigma), phosphatidylinositol 4-phosphate (PIP) (Boehringer Mannheim), or phosphatidylinositol 4,5-bisphosphate (PIP2) (Boehringer Mannheim) in a reaction containing [$^{32}$P]-$\gamma$-ATP as described (15). The *in vitro* $^{32}$P-labeled phosphoinositides, PIP2, and PIP3 then were used as standards for TLC analyses.

**Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay.** Log-phase growing MEF cells were seeded at a density of $5 \times 10^4$ cells per 12-mm round coverslip (Fisher Scientific). After attachment, cells were cultured in medium without serum for 72 hr. Cells were fixed in 3.7% formaldehyde/PBS, permeabilized in 0.2% Triton X-100/PBS and stained with rhodamine-phalloidin (Molecular Probes) in 10% normal goat serum/PBS, followed by counterstaining with TUNEL reaction mixture (Boehringer Mannheim). Cells were visualized by using fluorescence microscopy.

## RESULTS

**Generation of *Pten*$^{-/-}$ ES Cells and Characterization of Their Growth Properties.** We introduced a null mutation into the mouse *Pten* gene by homologous recombination in ES cells. Fig. 1*A* shows the targeting vector, pKO-1, in which both transcriptional and translational initiation sites and exons 1–3 of the *Pten* gene were deleted. By using an external probe, an 8.5-kb band corresponding to the targeted allele (Fig. 1*B*, lane 2) was detected in six of 200 G418$^r$ colonies. To facilitate the study of PTEN function at the cellular level, we generated *Pten*$^{-/-}$ ES cells by selecting with a higher dose of G418 (Fig. 1*B*, lane 3). In each of the following experiments, results were repeated and confirmed by using several independent ES clones to avoid potential clonal variations.

In the process of culturing *Pten*$^{+/+}$, *Pten*$^{+/-}$, and *Pten*$^{-/-}$ ES cells, we noticed that the *Pten*$^{-/-}$ ES cells appeared to reach high cell density faster. To determine whether *Pten*$^{-/-}$ cells had altered growth properties, we performed a growth competition experiment. Equal numbers of *Pten*$^{+/+}$ cells were mixed with *Pten*$^{+/-}$ or *Pten*$^{-/-}$ cells and cocultured. Cells were passed every 3 days to avoid growth saturation. During each passage, a fraction of the cell mix was harvested, and genomic DNA was prepared for Southern blot analysis to determine the representation of each cell type. As shown in Fig. 1*B* and quantified in Fig. 1*C*, the intensity of the 8.5-kb band, representing the *Pten* deletion allele (KO), increased significantly from passage 1 to passage 3 (Fig. 1*B*, lanes 5, 7, and 9) while the 23-kb fragment of the WT allele decreased correspondingly, indicating that the percentage of *Pten*$^{-/-}$ cells increased with each passage. At the end of the third passage (corresponding to 13–14 doublings of normal ES cells), the *Pten*$^{-/-}$ ES cells had outgrown the *Pten*$^{+/+}$ cells, as revealed by a dramatic reduction of the WT DNA band (Fig. 1*B*, lane 9) from the final cell population. As a control, when *Pten*$^{+/+}$ and *Pten*$^{+/-}$ cells were cocultured, the ratios between the KO and the WT alleles were largely unaltered during the consecutive passages (Fig. 1*B*, lanes 4, 6, and 8). Because under normal growth conditions, cell death is rare (< 1%), these results suggest that deletion of the *Pten* gene provides cells with a growth advantage, possibly by shortening the cell cycle time (see below).

*Pten*$^{+/+}$, *Pten*$^{+/-}$, and *Pten*$^{-/-}$ ES cells were compared for serum dependency. ES cells were cultured in parallel for 4 days in media containing reduced concentrations of serum. As shown in Fig. 1*D*, even in the medium without serum, *Pten*$^{-/-}$ ES cells were able to proliferate, and the total cell number was increased. In addition, *Pten*$^{-/-}$ ES cells survived better in reduced serum conditions. In serum-free medium, most of the *Pten*$^{+/+}$ and *Pten*$^{+/-}$ ES cells lost their viability in 2–3 days and detached from feeder layers (Fig. 1*E*, *a* and *b*). However, approximately 80% of *Pten*$^{-/-}$ ES cells remained and proliferated to form small colonies (Fig. 1*E*, *c* and *d*). Thus, the sustained growth of *Pten*$^{-/-}$ cells in the absence of serum could reflect a combination of increased cell survival and enhanced cell proliferation of *Pten*$^{-/-}$ ES cells.

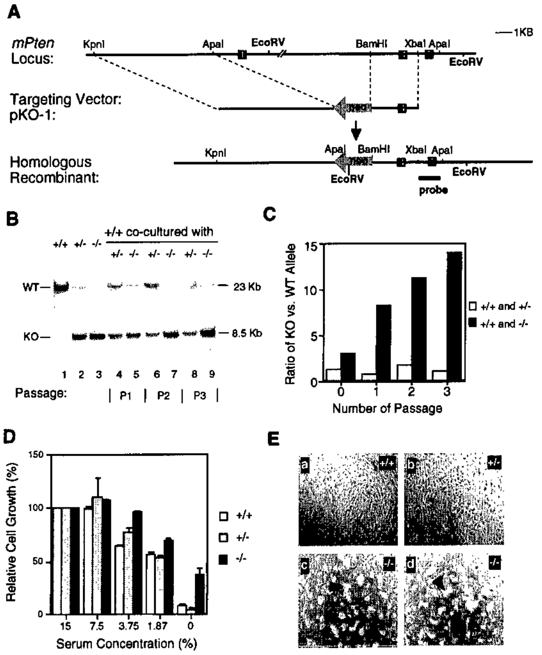

FIG. 1. Inactivation of the mouse *Pten* gene and the growth properties of *Pten*$^{-/-}$ ES cells. (*A*) A restriction map of the genomic region containing the *Pten* gene is shown at the top, with exons depicted. The targeting vector pKO-1 is shown in the middle. A restriction map of the predicted recombinant harboring the deleted allele is shown at the bottom. (*B*) Southern blot analysis. Lanes 1–3: DNA from *Pten*$^{+/+}$ (+/+, lane 1), heterozygous (+/−, lane 2), and homozygous (−/−, lane 3) ES cell cultures. Lanes 4–9: DNA from cocultured ES cells. An equal number ($4 \times 10^5$) of +/+ cells were cocultured with +/− cells in 33-mm dish (lanes 4, 6, and 8). Alternatively, an equal number of +/+ cells were cocultured with −/− cells (lanes 5, 7, and 9). DNA isolated from the indicated cultures were analyzed after one, two, or three passages. After *Eco*RV digestion, 23-kb and 8.5-kb bands, corresponding to the WT allele or the targeted allele (KO), respectively, could be detected by using an external probe. (*C*) Quantification of the amount of radioactivity in the hybridized restriction fragment corresponding to the WT allele (23 kb) or the *Pten* deletion allele (KO, 8.5 kb). The relative hybridization intensity of KO versus WT band in cocultures is presented. (*D*) ES cells ($1 \times 10^5$ cells/well in 24-well plate) were grown in media containing the indicated serum concentrations. Four days later, cells were counted. The relative cell growth was calculated by using cell numbers in 15% serum condition as 100%. Each value represents the average (±SD) obtained from a duplicate set of experiments. (*E*) WT (+/+, *a*), heterozygous (+/−, *b*) and two independent homozygous (−/−, *c* and *d*) ES cell lines were grown in serum-free medium for 4 days. Although no ES colonies, except the background feeder cell layers, were seen in the WT and heterozygous ES cultures (*a* and b, respectively), visible ES cell colonies (indicated by the arrowheads) were present in homozygous ES cultures (*c* and *d*). Scale bar: 200 $\mu$M.

**Advanced $G_1$ Cell Cycle Progression in *Pten*$^{-/-}$ Cells Correlates With Down-Regulation of p27$^{KIP1}$.** The ability of *Pten*$^{-/-}$ cells to proliferate under reduced serum conditions and their higher growth rate raised the possibility that deletion of the *Pten* gene may affect cell cycle progression. Our recent studies indicated that transient expression of the *PTEN* gene in a human glioblastoma cell line caused $G_1$ cell cycle arrest, suggesting that

PTEN may play a role in the $G_1$/S transition (9). These observations prompted us to examine whether *Pten*$^{-/-}$ cells have an accelerated cell cycle progression from $G_1$ into S phase.

ES cells are rapidly proliferating cells. Under normal growth conditions, we observed that about 60–70% of ES cells were in S phase. Because ES cells do not exhibit contact inhibition and respond poorly to serum deprivation, we used the mitotic shake-off method (12) for synchronization. Mitotic cells were collected after colcemid treatment and then released into fresh medium. S-phase entry was monitored by incorporation of [$^3$H]thymidine. Consistent with a previous report (12), [$^3$H]thymidine incorporation occurred about 3 hr after release from the mitotic block (Fig. 2*A*), suggesting that ES cells have a $G_1$ phase of about 2–3 hr. *Pten*$^{-/-}$ cells reproducibly showed an earlier (approximately 0.5–1 hr advance) and more synchronized entry into S phase compared with *Pten*$^{+/+}$ cells. Because the synchronized ES cells have a cell cycle time of approximately 9–10 hr, the advanced S-phase entry we observed in *Pten*$^{-/-}$ cells shortened the cell cycle time by 5–10%, which could have a significant impact on the growth rate of *Pten*$^{-/-}$ cells.

To determine the cause of early S-phase entry in PTEN-deficient cells, we examined the levels of $G_1$ cell cycle regulators, including two major $G_1$ CDK inhibitors, p21$^{CIP1/WAF1}$ and p27$^{KIP1}$. p21$^{CIP1/WAF1}$ and p27$^{KIP1}$ are involved in $G_1$/S progression, and their levels are known to be regulated by extracellular stimuli (16). Consistent with previous reports (17), p21 was not detectable in ES cells (data not shown), suggesting that p21 is not the major $G_1$ CDK inhibitor in ES cells. However, p27 was readily detectable in ES cells and its levels were oscillated in the cell cycle (Fig. 2*B*). In *Pten*$^{+/+}$ cells, p27 levels were low during mitosis (0 hr), transiently increased as cells entered the $G_1$ phase (2–3 hr), modestly reduced at the 4-hr time point, and returned to the basal level after 6 hr. In *Pten*$^{-/-}$ cells, however, both basal (0 hr) and induced levels of p27 during $G_1$ phase (2 and 3 hr) were significantly reduced. In addition, down-regulation of p27 occurred more rapidly in *Pten*$^{-/-}$ cells. By 4 hr, nearly 75% reduction in the p27 level was observed. As a control, the levels of cyclin D1, a major $G_1$ cyclin, were comparable in both *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells and were relatively constant throughout the cell cycle (Fig. 2*B*).

The binding of p27 to the $G_1$ cell cycle kinases, such as cyclin D/CDK4 or CDK6 and cyclin E/CDK2, leads to the inhibition of the activities of these kinases (16). To examine the effects of p27 reduction in *Pten*$^{-/-}$ cells on $G_1$ cell cycle kinase activity, we compared the activities of cyclin E/CDK2 complexes isolated from the synchronized *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells at various time points after mitotic shake-off. As shown in Fig. 2*C* and quantified in Fig. 2*D*, the loss of PTEN led to an increased level of cyclin E-associated kinase activity, which paralleled the decreased levels of p27 in the *Pten*$^{-/-}$ cells. These studies suggest that the down-regulation of p27 in *Pten*$^{-/-}$ cell may lead to enhanced activation of $G_1$ cell cycle kinases, which in turn promote advanced S-phase entry.

We also examined the p27 level in asynchronously growing cells. In *Pten*$^{-/-}$ cells, there is a reduction of p27 level by 3- to 4-fold as compared with *Pten*$^{+/+}$ cells (Fig. 2*E*). In contrast, the levels of other $G_1$ cell cycle regulators, such as cyclin D1, cyclin E, cyclin A, CDK2, as well as cyclin E-associated CDK2 or cyclin A-associated CDK2, did not show significant alteration (Fig. 2*E*). These results suggest that p27 is a selective target for the signaling pathway regulated by the PTEN tumor suppressor.

To address the question of whether the down-regulation of p27 in *Pten*$^{-/-}$ cells occurs at transcriptional or posttranscriptional levels, we compared the 27 mRNA level in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells by Northern blot analysis. We found that the level of p27 mRNA was not affected by PTEN status (Fig. 2*F*), suggesting that p27 is modulated by PTEN-regulated signaling pathway at the posttranscriptional level. In addition, the mRNA levels of cyclin D, cyclin E, cyclin A, or CDK2 were not affected by the PTEN status (Fig. 2*F*). Together, these data indicate the loss of PTEN affects cell cycle progression, and one selective target for this process is the down-regulation of p27 at its protein level.

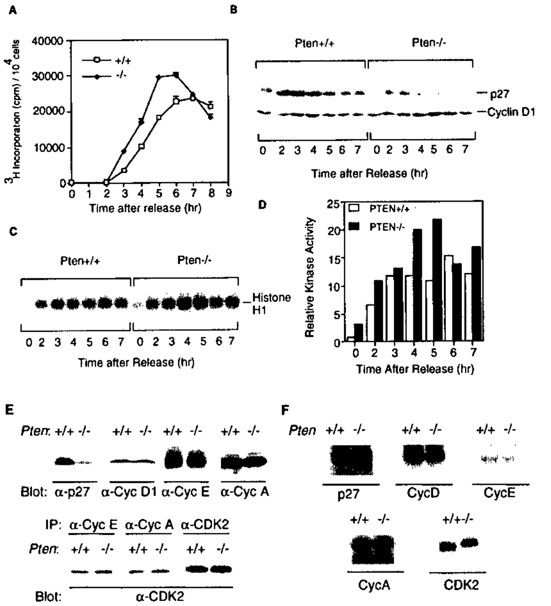

FIG. 2. Cell cycle progression and p27$^{KIP1}$ levels in synchronized and asynchronous *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells. (*A*) [$^3$H]thymidine incorporation after release from the colcemid block. For each time point, cells ($1 \times 10^5$) were pulse-labeled with [$^3$H]thymidine (1 $\mu$Ci/ml) for 1 hr before harvesting. [$^3$H]thymidine incorporation was measured. Each value represents the average (+SD) obtained from duplicate samples. (*B*) Western blot analysis of p27$^{KIP1}$ and cyclin D1 levels after release from colcemid block. Approximately $1 \times 10^6$ cells were seeded for each time point, and cell lysates (50 $\mu$g each) were examined by Western blot analysis with anti-p27 or anti-cyclin D1 antibody, respectively. (*C* and *D*) Histone H1 kinase activity assay. Cells were synchronized as described in *B*. Cyclin E/CDK2 complex was immunoprecipitated with anti-cyclin E antibody from cell lysates (300 $\mu$g each) and assayed for *in vitro* kinase activity by using histone H1 as substrate and $^{32}$p-[γ]-ATP. The relative kinase activity was obtained after quantification of the $^{32}$P-label incorporated into histone H1 by PhosphorImager. (*E*) Western blot analysis of various cell cycle regulators. Cell lysates from log-phase growing *Pten*$^{+/+}$ or *Pten*$^{-/-}$ cells (50 $\mu$g each) were analyzed by Western blot analysis with antibodies specific for p27$^{KIP1}$ or cyclin D1 (*Upper*). To examine the level of cyclin E or cyclin A, cell lysates (2 mg each) were immunoprecipitated with anti-cyclin E or anti-cyclin A antibodies, respectively, followed by Western blot analysis with the corresponding antibodies (*Upper*). (*Lower*) Cell lysates (2 mg each) were immunoprecipitated with antibodies for cyclin E, cyclin A, or CDK2, and analyzed with Western blot analysis with anti-CDK2 antibody. (*F*) Northern blot analysis. Total RNA (5 $\mu$g each) harvested from log-phase growing *Pten*$^{+/+}$ or *Pten*$^{-/-}$ cells were subjected to Northern blot analysis using p27, cyclin D, cyclin E, cyclin A, or CDK2 cDNA probe, respectively.

**PTEN Down-Regulates PI3 Kinase Signaling by Controlling PIP3 Accumulation.** Several signaling pathways are required for cells to progress from $G_1$ to S phase. Among them, PI3 kinase plays a major role (18). PI3 kinase regulates the production of PIP3, which acts as a second messenger of the PI3 kinase signaling pathway. PTEN can dephosphorylate PIP3 *in vitro* (6), and overexpression of the PTEN gene in human glioblastoma cells results in inhibition of Akt/PKB, a downstream target of PI3 kinase (9). These observations suggest that PTEN potentially may regulate the PI3 kinase signaling pathway.

By using genetically defined *Pten*$^{-/-}$ and *Pten*$^{+/+}$ but otherwise isogenic cells, we examined the levels of PIP3 in cells that have been stimulated with IGF-I. Fig. 3*A* shows a representative result from three independent experiments. *Pten*$^{+/+}$ cells were less sensitive to IGF-I stimulation, and their PIP3 levels were significantly lower than that of *Pten*$^{-/-}$ cells. Five minutes after IGF-I stimulation, only a modest increase in PIP3 level could be detected in *Pten*$^{+/+}$ cells. However, an approximately 2-fold increase of PIP3 (Fig. 3*B*) over the *Pten*$^{+/+}$ level could be detected in *Pten*$^{-/-}$ ES cells. Even 20 min after stimulation, the PIP3 level in *Pten*$^{-/-}$ ES cells was still very high. In contrast, the levels of PIP and PIP2 were largely unaffected by PTEN deficiency (Fig. 3*A*). The basal level (without IGF-I stimulation) of PIP3 is comparable in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells (data not shown). These results suggest that both the magnitude and duration of PIP3 accumulation after IGF-I stimulation were significantly higher in the *Pten*$^{-/-}$ ES cells. This increase in the PIP3 level in *Pten*$^{-/-}$ ES cells is quite reproducible and is comparable to the elevated PIP3 level induced by the overexpression of a constitutive active form of PI3 kinase (form p110*), as reported (19). These findings suggest that PIP3 is an intracellular target of PTEN.

**Increased Phosphorylation and Activation of PKB/Akt in *Pten*$^{-/-}$ ES Cells Correlates with Cell Proliferation and Down-Regulation of p27$^{\mathrm{KIP1}}$.** PKB/Akt, a growth factor-regulated serine/threonine kinase, is one of the best-characterized downstream targets of PIP3. Akt is activated by its association with PIP3, which facilitates Akt phosphorylation and activation by the upstream kinases (PDK1 and PDK2) (20). We examined Akt phosphorylation in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells after IGF-I stimulation. In *Pten*$^{+/+}$ ES cells, IGF-I induced a transient but modest phosphorylation of Akt on serine-473, which can be detected by a specific diagnostic antibody (Fig. 4*A*, *Left*). Akt phosphorylation reached its highest level around 20–40 min and subsequently was down-regulated by 90 min. In *Pten*$^{-/-}$ ES cells, both the basal level and the magnitude of Akt phosphorylation were significantly increased (Fig. 4*A*, *Right*). The duration of Akt phosphorylation also was prolonged, as no significant down-regulation of Akt phosphorylation could be observed in *Pten*$^{-/-}$ cells even 90 min after IGF-I stimulation. The sustained high level of Akt phosphorylation in *Pten*$^{-/-}$ cells suggests that the increase in PIP3 accumulation caused by PTEN deficiency is sufficient to induce the activation of Akt.

Because the selective growth advantage of *Pten*$^{-/-}$ ES cells was observed under normal culture conditions, we also examined whether the steady state of phosphorylated Akt is affected by the PTEN deficiency. As shown in Fig. 4*B*, we found that the level of Akt phosphorylation was very sensitive to the dosage of the *Pten* gene. The phosphorylated form of Akt could be detected in *Pten*$^{+/+}$ cells. In *Pten*$^{+/-}$ cells, a noticeable increase in Akt phosphorylation was observed. In *Pten*$^{-/-}$ cells, this increase became even more dramatic (Fig. 4*B*). Such changes of Akt status occurred only at the level of phosphorylation and therefore its activation; no changes in the protein level of Akt could be detected in these assays (Fig. 4*B*). We further examined whether Akt phosphorylation correlated with the growth status of the cells. As shown in Fig. 4*C*, in the lysates prepared from actively growing cells, there was a significant increase of Akt phosphorylation in two independent *Pten*$^{-/-}$ ES clones as compared with *Pten*$^{+/+}$ cells. This increase became less obvious when cells were harvested upon reaching confluence (Fig. 4*C*). In parallel, we also examined the level of p27$^{\mathrm{KIP1}}$. Similar to changes of phosphorylation of Akt, the difference of the p27 levels in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells was most pronounced in log-phase growing cells (Fig. 4*C*). As a control, the levels of cyclin D1 were independent of the PTEN status and the growth state of the cell (Fig. 4*C*). These observations raise the possibility that Akt, or other molecules activated by PIP3, may be involved in down-regulation of p27$^{\mathrm{KIP1}}$ and promoting cell proliferation.

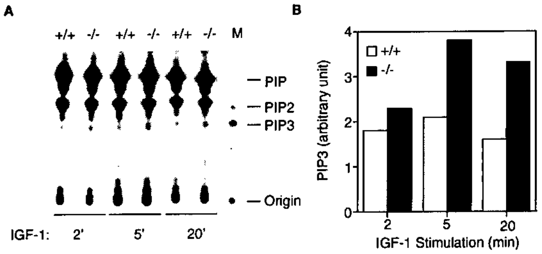

FIG. 3. PIP3 accumulation in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ cells. (*A*) PIP3 levels in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ ES cells after IGF-I stimulation. Cells were starved in a serum-free medium for 16 hr, and then labeled with [$^{32}$P]orthophosphate (0.5 mCi/ml) for 2 hr. Cells then were stimulated by IGF-I (1 μg/ml) for 2, 5, or 20 min before harvesting. Phospholipids were extracted and analyzed on a TLC plate. Assignment of PIP, PIP2, and PIP3 was done according to *in vitro* $^{32}$P-labeled phosphoinositides standards (see *Materials and Methods*). In lane M, [$^{32}$P]-labeled PIP3 is shown as a marker. (*B*) Quantitation of PIP3 levels in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ ES cells after IGF-I stimulation. The amount of radioactivity corresponding to PIP3 was measured with a PhosphorImager and presented as an arbitrary unit.

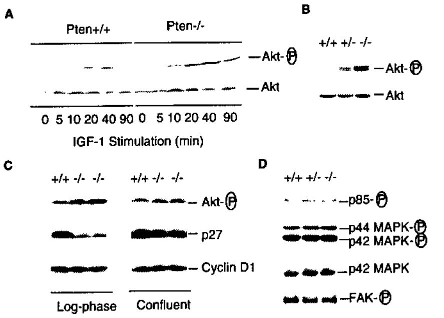

FIG. 4. Phosphorylation status of Akt, PI3 kinase, MAPK, and FAK, and the levels of p27 in *Pten*$^{+/+}$, *Pten*$^{+/-}$, or *Pten*$^{-/-}$ ES cells. (*A*) Phosphorylation status of Akt after IGF-I stimulation. *Pten*$^{+/+}$ and *Pten*$^{-/-}$ ES cells were passed twice without feeders to reduced background. Cells were serum-starved for 34 hr, then stimulated by IGF-I (1 μg/ml) for indicated time periods. Cell lysates (25 μg each) were examined by Western blot analysis with antibodies against phospho-Akt (serine-473) or Akt, respectively. (*B*) Phosphorylation status of Akt in actively growing ES cells. Log-phase growing *Pten*$^{+/+}$, *Pten*$^{+/-}$, or *Pten*$^{-/-}$ ES cells were harvested, and the cell lysates were analyzed with antibodies against phospho-Akt or Akt, respectively. (*C*) Akt, p27$^{\mathrm{KIP1}}$, and cyclin D1 levels in cells from different proliferation states. Cells were harvested from either log-phase cultures (*Left*) or confluent cultures (*Right*). Cell lysates (50 μg each) were examined by Western blot analysis with antibody specific for phospho-Akt, p27, or cyclin D1, respectively. (*D*) Phosphorylation status of PI3 kinase, MAPK, and FAK. Cell lysates were prepared from log-phase growing cells. To determine the phosphorylation status of the p85 subunit of PI3 kinase and FAK, cell lysates (500 μg each) were immunoprecipitated with antiphosphotyrosine antibody 4G10 followed by Western blot analysis with anti-p85 or anti-FAK antibody, respectively. To detect phosphorylated p42 and p44 MAPK, cell lysates (50 μg each) were examined by Western blots analysis with an antibody against phospho-MAPK. As a control, a duplicate filter was analyzed in parallel with an antibody for p42 MAPK.

To examine whether the increased PIP3 accumulation and Akt activation in *Pten*$^{-/-}$ cells is caused by up-regulation of PI3 kinase, we analyzed the tyrosine phosphorylation status of PI3 kinase itself in *Pten*$^{-/-}$, *Pten*$^{+/-}$, and *Pten*$^{+/+}$ cells. The PI3 kinase, the p85/p110 heterodimer, is activated by tyrosine phosphorylation on the p85 regulatory subunit (18). No significant alteration on p85 tyrosine phosphorylation could be detected in *Pten*$^{-/-}$ cells (Fig. 4*D*). These results suggest that PTEN functions downstream of PI3 kinase and that the elevated PIP3 level in *Pten*$^{-/-}$ ES cells is likely caused by impaired dephosphorylation of PIP3 by loss of PTEN rather than increased production by PI3 kinase.

To determine whether PTEN deficiency affects signaling molecules other than Akt, we examined phosphorylation status of MAPK and FAK. Our experiment revealed that the tyrosine phosphorylation and thus the activated forms of MAPK were not affected by the *Pten* deletion (Fig. 4*D*). It was reported that FAK could interact with and be dephosphorylated by PTEN in cells overexpressing PTEN (21). However, no significant difference in FAK phosphorylation could be detected among *Pten*$^{-/-}$, *Pten*$^{+/-}$, and *Pten*$^{+/+}$ cells (Fig. 4*D*). Thus, our studies suggest that PTEN selectively regulate the PIP3/Akt signaling pathway.

**An Increased Akt Activation in *Pten*$^{-/-}$ MEF Cells Leads to Increased Phosphorylation of Bad and Cell Survival.** ES cells are stem cells and represent the most undifferentiated cell type with high proliferative potential. Because of the unique status of ES cells, we were interested in determining whether inactivation of PTEN produced similar effects (e.g., increased growth rate and activation of Akt) in more differentiated cell types such as MEF cells. To generate *Pten*$^{-/-}$ MEF cells, we injected several lines of *Pten*$^{-/-}$ ES cells into blastocysts, and *Pten*$^{-/-}$ MEF were obtained from embryonic day 14–15 chimeric embryos after G418 selection to eliminate WT MEF cells. Complete inactivation of PTEN was confirmed by both Southern (data not shown) and Western blot analyses (Fig. 5*A*). During the course of culturing *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEF cells, we did not observe significant differences in the rate of growth, nor at the level of p27$^{\mathrm{KIP1}}$ (data not shown), possibly because of the differences in the cell cycle checkpoint control mechanism between ES and MEF cells (see *Discussion*).

Similar to *Pten*$^{-/-}$ ES cells, *Pten*$^{-/-}$ MEF cells contained a modest increase in PIP3 level (data not shown) and a significant enhancement in phosphorylation of Akt, as compared with their *Pten*$^{+/+}$ counterparts. Again, the total protein level of Akt was not affected (Fig. 5*A*). In addition, no detectable changes were observed for the level and phosphorylation status of MAPK (data not shown).

To demonstrate the causal relationship between the loss of PTEN and activation of Akt, we reintroduced either the WT *PTEN* gene or its mutant derivative *PTEN* CS back into *Pten*$^{-/-}$ cells by the retrovirus-mediated expression system. The *PTEN* CS mutant contains a substitution of the cysteine 124 with serine at the phosphatase signature motif and is catalytically inactive (3). Although Akt phosphorylation in *Pten*$^{-/-}$ MEF cells was significantly decreased upon reintroduction of the WT *PTEN* gene, no difference could be detected when *PTEN* CS was overexpressed (Fig. 5*A*, *Right*). These data reinforce the idea that Akt phosphorylation level depends on the *PTEN* gene dosage and that the phosphatase activity of PTEN is required for Akt inactivation.

Activation of Akt is essential for cell survival after growth factor withdrawal (20). To determine whether the increased Akt activity by PTEN deletion is sufficient to trigger downstream events, especially the growth survival effect of Akt, we examined the phosphorylation status of Bad, a member of the Bcl2 family. Bad can be phosphorylated by Akt, and such phosphorylation causes the loss of pro-apoptotic activity of Bad (22, 23). As shown in Fig. 5*B*, *Pten*$^{-/-}$ cells contained higher levels of phosphorylated Bad as compared with its *Pten*$^{+/+}$ counterparts, either in the presence or the absence of IGF-I stimulation. These data suggest that the Akt-controlled anti-cell death pathway is up-regulated and activated in *Pten*$^{-/-}$ cells.

To examine whether the increased phosphorylation of Akt and Bad affects MEF cell survival, we subjected *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEF cells to serum withdrawal. At different time points during serum starvation, cells were harvested and analyzed by two different procedures. First, we stained cells with propidium iodide and determined cell viability by fluorescence-activated cell sorting analysis. As summarized in Fig. 5*C*, the WT and *Pten*$^{-/-}$ MEF cells had significantly different survival rates in response to serum starvation. After serum starvation for 4 days, death occurred in more than 60% of WT cells, whereas fewer than 30% of *Pten*$^{-/-}$ cells underwent apoptosis. Second, TUNEL assays were performed to detect apoptotic cells by *in situ* staining. No significant cell death occurred in either population when cells were cultured under optimal growth conditions (Fig. 5*D*, *a* and *b*). However, 72 hr after serum withdrawal, a high incidence of apoptosis was observed in *Pten*$^{+/+}$ but not in *Pten*$^{-/-}$ MEF cultures (Fig. 5*D*, *c* and *d*). These data suggest that inactivation of PTEN leads to up-regulation of the AKT pathway and prevents cells from apoptotic death.

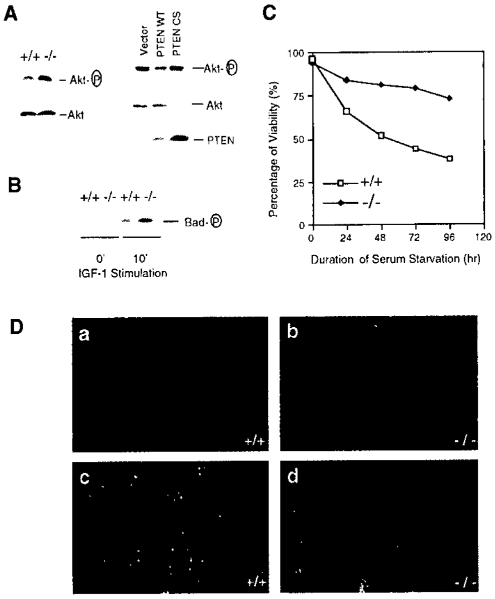

FIG. 5. Increased phosphorylation of Akt and Bad promotes *Pten*$^{-/-}$ MEF cells survival. (*A*, *Left*) MEF cell lysates were prepared and subjected to Western blot analyses as described in the legend of Fig. 4*B*. (*A*, *Right*) *Pten*$^{-/-}$ MEF cells were infected with retroviruses carrying empty vector, the WT PTEN, or the PTEN CS mutant. Cells were harvested 48 hr postinfection. Cell lysates (50 $\mu$g each) were subjected to Western blot analysis with antibodies specific for phospho-Akt or Akt, respectively. A duplicate filter also was analyzed with anti-PTEN antibody. (*B*) MEF cells were serum-starved for 16 hr, then labeled with [$^{32}$P]orthrophosphate for 4 hr. Cells then were stimulated with IGF-I (1 $\mu$g/ml) for 10 min before harvest. Cell lysates were subjected to immunoprecipitation with anti-Bad antibody, and the immunoprecipitates were analyzed by SDS/PAGE and autoradiography. (*C*) Propidium iodide staining. *Pten*$^{+/+}$ or *Pten*$^{-/-}$ MEF cells were seeded in serum-free medium. At the indicated time, cells (both adherent and in suspension) were collected and stained with isotonic propidium iodide solution. Percentage of cell viability, determined by using fluorescence-activated cell sorting analysis, is presented. (*D*) TUNEL assay. Log-phase *Pten*$^{+/+}$ (*a* and *c*) and *Pten*$^{-/-}$ (*b* and *d*) MEF cells were grown with (*a* and *b*) or without (*c* and *d*) serum for 72 hr. Cells were stained with TUNEL reaction mix (green) and counterstained with rhodamine-phalloidin (red). Apoptotic cells were indicated by positive staining with both TUNEL reaction mix and pholloidin dye (yellow).

## DISCUSSION

By using a genetically defined system, we have demonstrated that PTEN negatively regulates PIP3 and Akt signaling pathway. Several lines of evidence suggest that accumulation of PIP3 in *Pten*$^{-/-}$ cells is caused by the loss of PTEN phosphatase activity. First, we have shown that the activity of PI3 kinase, the enzyme that specifically produces PIP3, was not altered in *Pten*$^{-/-}$ ES cells. Thus the observed higher PIP3 levels in *Pten*$^{-/-}$ cells is likely caused by the decreased dephosphorylation of PIP3. Second, we showed that by reintroducing the WT *PTEN* gene into *Pten*$^{-/-}$ MEF cells, we could reverse PIP3-dependent Akt activation. Such a reversion requires the phosphatase activity of PTEN, suggesting that PTEN is likely to be a bona fide phosphatase for PIP3. Our data are further supported by the results from previous experiments, which showed that PTEN could act as a specific phosphoinositide 3-phosphatase *in vitro* and overexpression of PTEN in 293 cells resulted in decreased PIP3 levels (6).

We have further demonstrated that Akt/PKB, a downstream target for PIP3 signaling, is up-regulated in PTEN-deficient cells. Akt phosphorylation and activation depend on the dosage as well as the phosphatase activity of the Pten gene product. In log-phase growing *Pten*$^{-/-}$ cells, the steady-state level of Akt phosphorylation is 3- to 4-fold higher than *Pten*$^{+/+}$ cells. Moreover, Akt activation directly correlates with the proliferation state of the cells. A direct role of Akt in cell cycle progression has been suggested by the recent observation that expression of a constitutive activated Akt in mouse macrophage cells or rat fibroblasts can trigger S-phase entry in the absence of serum growth factors (24, 25). Together, these results indicate that Akt may be a critical molecule involved in the regulation of normal cell growth.

$p27^{KIP1}$ has been proposed to prevent unscheduled activation of cyclin-CDK complexes in $G_1$ phase (16). Several reports (9, 26–28) suggest that PI3 kinase signaling may be involved in the down-regulation of p27. In NIH 3T3 cells, expression of a dominant-negative form of Ras, probably through inhibition of PI3 kinase, can block cell cycle progression at mid or late $G_1$ phase (26, 27). In aortic smooth muscle cells, treatment of cells with wortmannin, a pharmacologic inhibitor for PI3 kinase, also blocked $G_1$ cell cycle progression (28). In both systems, the $G_1$ cell cycle block was accompanied by failure to down-regulate p27 (26–28). We recently observed that in U87MG human glioblastoma cells transient expression of PTEN leads to significant up-regulation of p27 and $G_1$ cell cycle arrest (9). However, it is unclear from these experiments whether the increased $p27^{KIP1}$ level is the cause or the consequence of $G_1$ cell cycle arrest.

In this study, we have provided *in vivo* evidence that $p27^{KIP1}$ is a downstream target of the PIP3 signaling pathway and such regulation occurs at the posttranscriptional level. Our studies suggest that down-regulation of $p27^{KIP1}$ is likely to be a critical or even a rate-limiting step during $G_1/S$ transition in ES cells. We have attempted to reintroduce the *PTEN* gene into $PTEN^{-/-}$ ES cells to rescue the cell-cycle regulation defect. However, we failed to obtain stable cell clones that expressed the exogenous *PTEN* gene (data not shown). This failure was likely caused by the growth suppression effect of overexpressed PTEN in these cells, a phenomenon similar to that observed in human glioblastoma cells (7–9). It is interesting to point out that down-regulation of $p27^{KIP1}$ and advanced cell cycle progression are most notable in $Pten^{-/-}$ ES cells, but are less obvious in more differentiated MEF cells. Such differential cell responses may reflect the differences in intrinsic cell cycle control mechanisms between these two cell types. Down-regulation of $p27^{KIP1}$ by PIP3 signaling may be sufficient for cell cycle progression in ES cells, whereas in MEF cells additional signaling processes may be required. This scenario is consistent with tumorigenesis in animals and humans. Mice lacking $p27^{KIP1}$ suffer from multiple organ hyperplasia (29–31). A common feature among these organs is that their progenitor cells are undifferentiated and mitotically active. Many tumor cells also arise from undifferentiated progenitor cells, and the mutation in the PTEN gene in those cells may be sufficient to promote cell cycle progression. It is interesting to note that PTEN mutations frequently are observed in advanced human prostate cancer (32) and the levels of p27 inversely correlate with prognosis of prostate cancer (33). It is possible that the decreased $p27^{KIP1}$ level in prostate cancer tissues occurs as a consequence of impaired PTEN function.

While this manuscript was under review, Stambolic *et al.* (34) reported that immortalized $Pten^{3-5}$ (disruption of exons 3–5 of the murine *Pten* gene) MEF cells exhibited resistance to cell death stimuli, accompanied by increases of PIP3 level and Akt activation. Their conclusion that PTEN regulates apoptosis is consistent with part of the conclusion discussed in this paper. They also observed that a prominent feature in $Pten^{-/-}$ embryos was enhanced cell proliferation during early embryogenesis, although they had not provided a mechanistic explanation for this phenotype. Di Cristofano (10) *et al.* recently reported that PTEN is essential for embryonic development, and $Pten^{+/-}$ mice or chimaeric mice derived from $Pten^{+/-}$ ES cells show hyperplastic-dysplastic changes in prostate, skin, and colon, and the mice also develop germ cell, gonadostroma, thyroid, and colon tumors. These observations further highlight the importance of PTEN in regulation of cell proliferation, and our findings of PTEN deficiency having a profound effect on cell cycle progression provides a molecular basis for these phenotypes.

In summary, our studies reveal an insight into the mechanism by which PTEN functions as a tumor suppressor. By regulating PIP3 and Akt/PKB, PTEN modulates two fundamental cellular processes: cell cycle progression and cell survival. Alteration of either or both processes has long been implicated in the genesis of human cancer.

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Cell Biology

# PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces $G_1$ cell cycle arrest in human glioblastoma cells

**(tumor suppressor/phosphatase/signal transduction)**

DA-MING LI AND HONG SUN*

Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520

*Edited by Alfred G. Knudson, Jr., Institute for Cancer Research, Philadelphia, PA, and approved October 27, 1998 (received for review August 19, 1998)*

ABSTRACT **PTEN/MMAC1/TEP1 is a tumor suppressor that possesses intrinsic phosphatase activity. Deletions or mutations of its encoding gene are associated with a variety of human cancers. However, very little is known about the molecular mechanisms by which this important tumor suppressor regulates cell growth. Here, we show that PTEN expression potently suppressed the growth and tumorigenicity of human glioblastoma U87MG cells. The growth suppression activity of PTEN was mediated by its ability to block cell cycle progression in the $G_1$ phase. Such an arrest correlated with a significant increase of the cell cycle kinase inhibitor $p27^{KIP1}$ and a concomitant decrease in the activities of the $G_1$ cyclin-dependent kinases. PTEN expression also led to the inhibition of Akt/protein kinase B, a serine-threonine kinase activated by the phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathway. In addition, the effect of PTEN on $p27^{KIP1}$ and the cell cycle can be mimicked by treatment of U87MG cells with LY294002, a selective inhibitor of PI 3-kinase. Taken together, our studies suggest that the PTEN tumor suppressor modulates $G_1$ cell cycle progression through negatively regulating the PI 3-kinase/Akt signaling pathway, and one critical target of this signaling process is the cyclin-dependent kinase inhibitor $p27^{KIP1}$.**

Glioblastoma is the most common and malignant brain tumor characterized by aggressive growth and extensive vascularization through angiogenesis (1). A frequent genetic event in the progression of low-grade glioma to high grade, invasive glioblastoma is the loss of heterozygosity at chromosome 10q23 (2). A candidate tumor suppressor gene, PTEN or MMAC1, has been isolated from chromosome 10q23 locus by positional cloning (3, 4). In an independent approach to identify novel phosphatases, we have cloned TEP1, which is identical to PTEN or MMAC1 (5). The PTEN gene is mutated at high frequency in many primary human cancers and several familial cancer predisposition disorders (6). PTEN contains the sequence motif that is highly conserved in the members of the protein tyrosine phosphatase family. PTEN has been shown *in vitro* to possess phosphatase activity on phosphotyrosyl and phosphoseryl/threonyl-containing substrates (5, 7). More recently, PTEN has been shown, *in vitro*, to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate, a product of phosphatidylinositol 3-kinase (PI 3-kinase) (8). Consistent with the notion that the phosphatase activity of PTEN is required for the tumor suppressor activity, many cancer-derived mutations are mapped in its conserved catalytic domain. PTEN has been implicated in cellular processes such as cell migration and spreading (9). However, the exact function of PTEN in cell growth and tumorigenesis remains unclear.

In this study, we have investigated the function of the tumor suppressor protein PTEN in cell growth. We show that expression of PTEN potently suppresses the growth and tumorigenicity of human glioblastoma cells. Such effects are correlated with its ability to mediate $G_1$ cell cycle arrest and induce the cell cycle kinase inhibitor, $p27^{KIP1}$.

## MATERIALS AND METHODS

**Cell Culture.** U87MG (human glioblastoma) was obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum.

**Antibodies.** A synthetic peptide, CGGGDSDPENEPFDEDQHTQITKV (underlined letters corresponding to the residues 384–403 at the C terminus of PTEN) was synthesized and used to raise antibody in rabbits (YU102). The anti-PTEN antibody was affinity-purified with the synthetic peptide coupled to the SulfoLink gel (Pierce). Antibodies specific for $p27^{KIP1}$ (sc-528), cyclin E (sc-198), or cyclin D (R-124) were obtained from Santa Cruz Biotechnology, and anti-$p21^{CIP1/WAF1}$ (Ab-1) were from Oncogene Sciences. Anti-p42 mitogen-activated protein kinase (MAPK) antibody (B9) was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Akt/protein kinase B (PKB) and anti-phospho-MAPK antibodies were from New England Biolabs. Anti-cyclin-dependent kinase 2 (anti-CDK2) and anti-cyclin A antibodies were kindly provided by Hui Zhang at Yale University (10).

**Retrovirus Infection.** Bing cells or Bosc-23 cells were transfected with the retroviral plasmids, pBabePuro, pBabePuro-PTEN, or pBabePuro-PTEN(CS), and the retroviruses packaging and infection were carried out according to the methods described (11). For colony formation assay, an equal aliquot of the amphotropic viruses packaged in Bing cells were used to infect in duplicates U87MG or DLD1 cells. The infected cells were selected in medium containing 1 μg/ml puromycin. U87-EcoR cells were established from U87MG cells by using the murine ecotropic receptor-expressing retroviruses, which were produced in Bing cells after transfection with the pWZL-Neo-EcoR plasmid (12). The infected cells were selected in medium containing 400 μg/ml neomycin, and the drug-resistant colonies were pooled and expanded (U87-EcoR cells). To infect U87-EcoR cells, ecotropic retroviruses were packaged in Bosc-23 cells.

**Soft Agar and Nude Mice Studies.** Logarithmically growing cells ($1 \times 10^4$ cells) were plated as a single-cell suspension in 0.3% agarose in DMEM supplemented with 10% fetal bovine serum. Colonies were inspected under the microscope and

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Abbreviations: BrdUrd, bromodeoxyuridine; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B.

*To whom reprint requests should be addressed. e-mail: hong.sun@yale.edu.

photographed 4 weeks later. For tumor formation in nude mice assay, cells were trypsinized and resuspended in PBS, and 5 × $10^5$ cells (in 0.1 ml) were injected into each flank of the nude mice (The Jackson Laboratory, nu/nu). Tumors were monitored weekly after inoculation.

**Flow Cytometry, Immunoprecipitation, and Western Blot Analyses.** Fluorescence-activated cell sorting analysis was performed by using fluorescein-conjugated anti-bromodeoxyuridine (BrdUrd) antibody (Becton Dickinson) following the supplier's protocol. Immunoprecipitation, Western blot analysis, and histone H1 kinase assay were carried out as described (10).

## RESULTS

**PTEN Selectively Suppresses the Growth of Certain Tumor Cell Lines.** To investigate whether the loss of PTEN may provide a growth advantage to tumor cells that contain inactivating PTEN mutations, we examined the function of the PTEN gene in human glioblastoma cell line U87MG. U87MG has been shown to carry a deletion in the PTEN gene (3). Using an anti-PTEN antibody YU102, we found that U87MG cells did not contain any detectable PTEN protein, confirming that these cells are indeed deficient in PTEN (Fig. 1*C*). To investigate whether deletion of the PTEN gene confers any growth advantage, we reintroduced the wild-type PTEN gene into the U87MG cells and analyzed its effect on cell growth. To increase the gene delivery efficiency, the PTEN cDNA was cloned into a retroviral vector (pBabePuro), which carries the puromycin selection marker. The recombinant PTEN retroviruses, as well as the control (the empty retroviral vector) viruses, were produced from Bing cells as amphotropic viruses and used to infect the U87MG cells. After infection, stable colonies were selected in puromycin-containing medium and counted after 2–4 weeks. U87MG cells infected with the vector control retroviruses produced many large and densely growing colonies in 2 weeks in the selection medium. However, in PTEN retrovirus infected cells, few colonies were observed during the same time period. Very small colonies did appear in PTEN infected cells after a longer incubation (4 weeks) (Fig. 1*A Upper*). These observations suggest that PTEN exerted potent growth suppression in U87MG cells. In contrast, in human colon carcinoma cells, DLD1, there were much less differences in the number of drug resistant colonies between the vector control and PTEN virus infected cells (Fig. 1*A Lower*). The results from DLD1 cells also indicate that the titers for the vector control and PTEN-expressing viruses were similar. In addition to DLD1, a variety of other cell lines such as human colon carcinoma cells HCT116 and mink lung epithelial cells (MvLu), appeared to be relatively resistant to PTEN-mediated growth suppression (data not shown). These studies suggest that PTEN can suppress cell growth in a cell-type dependent manner. Our observations are consistent with the previous report showing that expression of the PTEN gene led to 60–70% reduction of cell numbers in PTEN-deficient human glioma cells in a transient transfection assay (13).

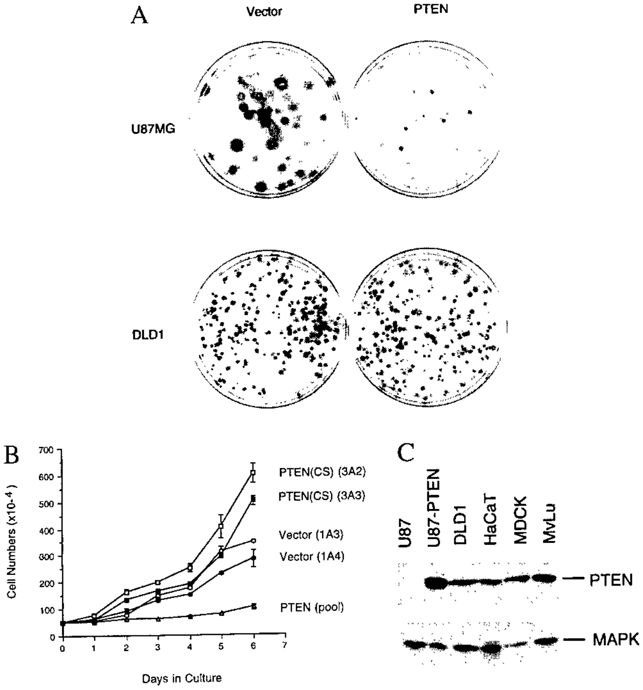

FIG. 1. Growth inhibition by PTEN. (*A*) PTEN selectively suppresses colony formation in U87MG cells. Glioblastoma U87MG (*Upper*) or colon carcinoma DLD1 (*Lower*) cells were infected with either empty-vector control (*Left*) or PTEN-expressing retroviruses (*Right*). The infected cells were then selected in puromycin containing medium for either 4 weeks (U87MG) or 2 weeks (DLD1). Cells were fixed, stained with Giemsa dye, and photographed. (*B*) PTEN expression inhibits U87MG cell doublings. Two independent stable U87MG cell clones that contain the empty vector (Vector, clones 1A3 and 1A4) and the catalytically inactive PTEN(CS) mutant [PTEN(CS), clones 3A2 and 3A3], or the pooled clones from U87MG derivatives expressing PTEN (PTEN, pool), were analyzed. 5 × $10^5$ cells from each cell line (in duplicates) were seeded onto culture dishes and the average cell numbers (with the standard deviation) were presented. (*C*) Western blot analyses. Cell lysates (50 μg each) from the indicated cell lines were examined by Western blot analysis with anti-PTEN antibody or anti-p42 MAPK antibody, respectively.

To further examine the growth properties of the PTEN-expressing cells, we recovered the drug resistant cell clones after retrovirus infection. Because the PTEN-retrovirus infected U87MG cells grow extremely slow, the drug resistant clones were pooled and cultured further. As the controls, we isolated cell colonies that were derived from cells infected either with retroviruses carrying the empty vector or with retroviruses expressing PTEN(CS), a mutant derivative of PTEN that contains Cys-124 to serine substitution at the phosphatase catalytic center. We have previously shown that the C124S mutation in PTEN completely abolished the phosphatase activity of the enzyme (5). To determine whether PTEN expression causes an increase of cell doubling time, we compared the growth rate of the control and PTEN-expressing cells. Equal number of cells from each cell line were plated in culture dishes and the number of cells were monitored every 24 hr after seeding. As shown in Fig. 1*B*, whereas the vector control or PTEN(CS)-expressing cells have a doubling time of 24–36 hr, the PTEN-expressing derivatives have a doubling time of ≈6 days. In addition, the PTEN(CS)-expressing cells appeared to grow to higher saturation density than the vector control cells, suggesting that the catalytically inactive PTEN(CS) mutant may provide a growth advantage to these cells.

To examine the level of PTEN expression in U87MG cells, we performed Western blot analysis with an anti-PTEN antibody. As shown in Fig. 1*C*, U87-PTEN stable cell lines express PTEN at a level that was comparable to those detected in a variety of human and rodent cell lines, including DLD1, HaCaT (human keratinocytes), Madin–Darby canine kidney (MDCK) epithelial cells and MvLu cells. As control, the level of p42 MAPK is also examined in parallel (Fig. 1*C*). DLD1, HaCaT, MDCK, and MvLu cells all contain active PTEN protein, as determined by the phosphatase activity assay of the anti-PTEN immunoprecipitates (data not shown). In addition, PTEN and PTEN(CS) were expressed at similar levels in U87MG derivatives (see Fig. 4*B*). These results further indicate that the expression of the wild-type PTEN gene confers a potent growth suppression effect in U87MG cells.

**PTEN Suppresses the Tumorigenicity of Human Glioblastoma U87MG Cells.** In addition to reduction in growth rate, expression of the PTEN gene in U87MG cells caused morphological changes. The vector control cells usually grow into aggregates to form "cell clumps" in culture (Fig. 2*A*). Similar morphology has also been observed in cells-expressing the catalytically inactive mutant, PTEN(CS) and the parental cells (data not shown). However, the PTEN-expressing cells showed a much flattened cell morphology and nonrefractile appearance (Fig. 2*A*). These observations suggest that the expression of PTEN profoundly alters the cell shape and/or cell–matrix interactions.

Changes in cell morphology have been shown to be associated with malignant transformation in many tumor cells. A hallmark of transformation is that transformed cells can grow in the absence of cell anchorage, as reflected by their ability to grow in soft agar and in nude mice. To investigate whether the morphological changes induced by PTEN expression correlates with a reduction in the transformation properties of these cells, we compared the ability of U87MG cells carrying either the empty vector, or expressing PTEN or the catalytically-inactive mutant PTEN(CS) to grow in soft agar and in nude mice. Whereas the vector control and PTEN(CS)-expressing cells readily grew in soft agar, the PTEN-expressing cells lost their ability to grow in soft agar (Fig. 2*B Upper*). We also assayed these cells for their ability to form tumors in nude mice. In mice injected with vector control cells, 6/6 injected sites grew tumors with average tumor size 1,766 mm$^3$ at 8 weeks after inoculation. But in mice injected with PTEN-expressing cells, 9 of 10 injected sites were tumor free when examined by dissection 15 weeks after inoculation, and 1 in 10 sites contained a very small tumor (tumor size <14 mm$^3$). In contrast, in mice injected with PTEN(CS)-expressing cells, 7 out of 8 sites grew tumors with average tumor size 5,960 mm$^3$ at 8-weeks. The more aggressive tumor growth was observed with the PTEN(CS)-expressing cells, suggesting that the catalytic inactive mutant phosphatase may have a growth promoting effect. A comparison of these mice at 8-weeks after inoculation is shown in Fig. 2*B* (*Lower*). These studies indicate that the expression of PTEN suppressed the anchorage-independent growth of U87MG cells in soft agar and their tumorigenecity *in vivo* and such function of PTEN is dependent on its phosphatase activity.

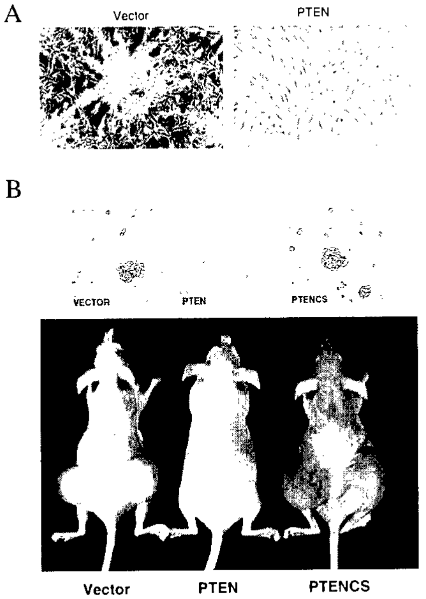

FIG. 2. Alteration of growth properties in PTEN-expressing U87MG cells. (*A*) Morphological changes. The vector control U87MG cells (*Left*) were highly refractile and many grew in "cell clumps." The PTEN-expressing cells (*Right*) were much flatter and non-refractile. Photographs were taken at the same magnification (×200). (*B*) PTEN suppressed cell growth in soft agar and in nude mice. (*Upper*) $10^4$ cells (a representation from duplicated samples) from U87MG cell clones that contain either the empty vector, or cells expressing PTEN or the PTEN(CS) mutant were assayed for their ability to grow in soft agar. Photographs were taken 4 weeks after seeding. (*Lower*) Tumor formation in nude mice. Cells ($5 \times 10^5$) were injected into each site in the nude mice (two sites per mouse) and mice were monitored weekly after inoculation. A photograph from representative mice 8 weeks after injection with the indicated cells is shown.

**PTEN Expression Leads $G_1$ Cell Cycle Arrest.** To examine the molecular mechanisms by which PTEN mediates growth suppression, we have examined the cell cycle effect after the transient PTEN-expression in U87MG glioblastoma cells. To improve the retrovirus gene delivery efficiency into human cells, we have established U87MG derivatives that stably express the murine ecotropic receptor (12). These cells, designated as U87-EcoR, could be infected with ecotropic retroviruses at >90% efficiency, as assayed using a control LacZ retrovirus (data not shown). Such an efficient gene delivery system allows us to analyze the effect of PTEN on the cell cycle in the total cell population.

To examine the effect of PTEN expression on cell cycle progression, U87-EcoR cells were infected with ecotropic retroviruses expressing either PTEN, the catalytically-inactive PTEN(CS) mutant, or an empty vector control. Forty-eight hours postinfection, cells were pulsed-labeled with BrdUrd, a thymidine analog. The cells were fixed, stained with fluorescein-conjugated anti-BrdUrd antibody and propidium iodide, and examined for cell cycle profile by using the fluorescence-activated cell sorting analysis method. As shown in Fig. 3*A*, expression of PTEN led to >50% reduction in the S phase cell population and a substantial increase in the $G_0/G_1$ population, as compared with that of the vector control. The cell cycle arrest caused by PTEN expression also depended on its phosphatase activity, as very little effect on the cell cycle profile was observed when the catalytically-inactive PTEN(CS) mutant was expressed (Fig. 3*A*).

**PTEN Expression Causes Inhibition of the $G_1$ CDKs.** In mammalian cells, cell cycle progression is controlled by the sequential activation of CDKs (14). To examine whether the observed $G_0/G_1$ cell cycle arrest by PTEN expression is accompanied by changes in the activities of the cell cycle regulatory machinery, we have examined the $G_1$ CDK activities. The cells were infected with either the vector control or PTEN retroviruses. Forty-eight hours after infection, CDKs were isolated from cell lysates by immunoprecipitation by using specific CDK or cyclin antibodies. The isolated CDKs were assayed for their kinase activities *in vitro* by using histone H1 as a substrate. As shown in Fig. 3*B*, in cells expressing PTEN, there was a significant decrease in the CDK2 kinase activity, as compared with the vector control cells. Because CDK2 is normally associated with cyclin E and cyclin A, which regulate the $G_1/S$ transition, we also assayed for the kinase activity associated with cyclin E and cyclin A. Both cyclin E- and cyclin A-associated kinase activities were also significantly reduced by the expression of PTEN (Fig. 3*B*). In contrast, in cells expressing the catalytically inactive PTEN(CS) mutant, no reduction in CDK2, cyclin E/CDK2, or cyclin A/CDK2 kinase activities was observed (Fig. 3*B*). These studies indicate that PTEN expression leads to inhibition of the $G_1$ cyclin-dependent kinase activities, and such inhibition depends on the phosphatase activity of PTEN. Due to technical difficulties (15), we have not been able to assay the cyclin D/CDK4 or cyclin D/CDK6-associated kinase activities in these cells.

**PTEN Expression Induces Accumulation of $p27^{KIP1}$.** The functions of several tumor suppressors have been shown to be mediated in part through the regulation of the CDK inhibitors (14). To investigate whether CDK2 inhibition observed in PTEN-expressing cells were associated with alteration in the

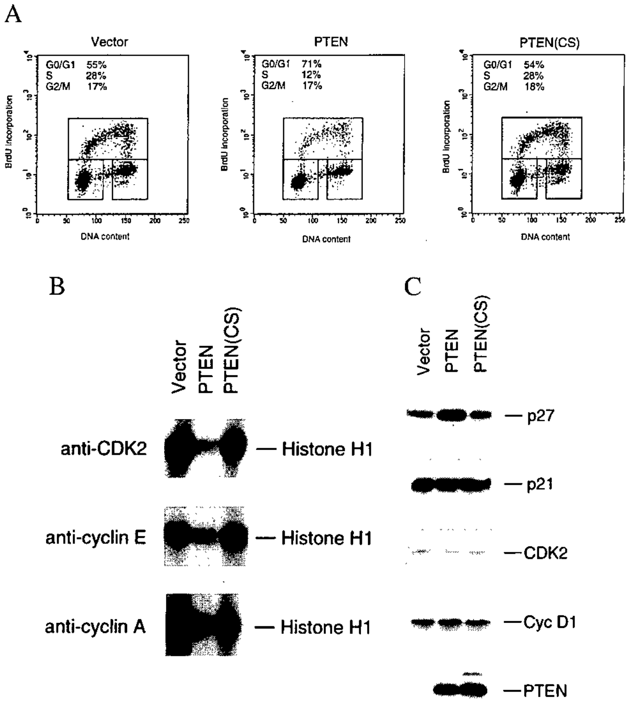

FIG. 3. Transient expression of PTEN leads to $G_1$ cell cycle arrest and causes changes in the activities of cell cycle regulators. (*A*) Flow cytometry analysis of cell cycle distribution. U87-EcoR cells were infected with retroviruses carrying either the empty vector, PTEN or PTEN(CS). Infected cells were harvested 48 hr later. Before harvesting, cells were pulse-labeled with BrdUrd for 3 hr. BrdUrd incorporation was analyzed by fluorescein-conjugated anti-BrdUrd antibody (vertical axis) and DNA-content was measured by propidium iodide staining (horizontal axis). The percentage of cells in S phase (upper boxes), $G_0/G_1$ phase (lower-left boxes), $G_2/M$ phase (lower-right boxes) are indicated. Similar results were obtained from five independent experiments and one of them is shown here. (*B*) Histone H1 kinase activity assay. Cells were infected with either vector, PTEN or PTEN(CS)-expressing retroviruses and harvested 48 hr later. Immunoprecipitation was carried out by using either anti-CDK2, anti-cyclin E, or anti-cyclin A antibody from each cell lysate (350 μg), respectively. The immunoprecipitated CDKs were assayed for kinase activity by using histone H1 as the substrate. Similar results were obtained from the duplicates of the immunoprecipitation and kinase assay (one set of the duplicates is presented). (*C*) Western blot analysis. Cell lysates (50 μg each) from cells infected with either vector, PTEN, or PTEN(CS)-expressing retroviruses were subjected to Western blot analysis with specific antibodies against $p27^{KIP1}$, $p21^{CIP1/WAF1}$, CDK2, cyclin D1, or PTEN, respectively.

levels of CDK inhibitors, we examined the levels of $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ in cells infected with retroviruses either carrying no insert (vector control), PTEN, or its mutant derivative PTEN(CS). As shown in Fig. 3*C*, expression of PTEN led to a significant elevation of p27, as revealed by Western blot analysis with anti-p27 antibody. The accumulation of p27 also depended on the phosphatase activity of PTEN, because the catalytically inactive PTEN(CS) mutant did not cause p27 accumulation. In these experiments, the increase of p27 could be observed in either direct Western blot analysis of the cell lysates (Fig. 3*C*) or immunoprecipitation followed by Western blot analysis with anti-p27 antibody (data not shown). We have consistently observed increase of p27 level after PTEN expression in five independent experiments, with an average increase of ≈3-fold. In parallel, we have also examined the level of $p21^{CIP1/WAF1}$ in these cells by Western blot analysis with anti-p21 antibody. In contrast with the p27 results, there was very little change in the p21 protein level in cells expressing PTEN or its PTEN(CS) mutant, as compared with vector control (Fig. 3*C*). In addition, PTEN expression had little effect on the protein levels of CDK2 or cyclin D1 (Fig. 3*C*). Because the cells infected with PTEN-retroviruses contain the similar levels of cyclin D1 as the control virus-infected cells and because cyclin D1 is known to be expressed at high levels at $G_1$ but very low in $G_0$ phase cells, the PTEN-expressing cells are likely to be arrested in the $G_1$ phase of the cell cycle. The expression of PTEN and PTEN(CS) proteins were confirmed by Western blot analysis with anti-PTEN antibody (Fig. 3*C*).

**PTEN Expression Inhibits the Akt/PKB Signaling Pathway.** Down-regulation of $p27^{KIP1}$ in the $G_1$ phase has been shown in certain cells to involve the function of PI 3-kinases (16, 17). PI 3-kinases regulate the levels of inositol phospholipids, which act as second messengers (18). One of the best characterized downstream targets activated by PI 3-kinase signaling is Akt/PKB, a serine/threonine kinase encoded by a protooncogene (19). To investigate if PI 3-kinase downstream signaling is involved in PTEN-mediated growth suppression, we examined the status of Akt in cells infected with retroviruses carrying PTEN, PTEN(CS), or the vector control. As shown in Fig. 4*A*, expression of PTEN, but not its catalytically-inactive mutant PTEN(CS), led to a substantial reduction in the amount of the phosphorylated, active forms of Akt, as determined by using an antibody that specifically recognizes the Ser-473-phosphorylated Akt (Fig. 4*A*). The reduction in Akt phosphorylation signal is not due to the decrease in protein level, because the total protein level of Akt was even slightly elevated by PTEN expression, as revealed by Western blot analysis using antibody that recognized both phosphorylated and unphosphorylated forms of Akt (Fig. 4*A*). Dephosphorylation of Akt was also accompanied by shift of its

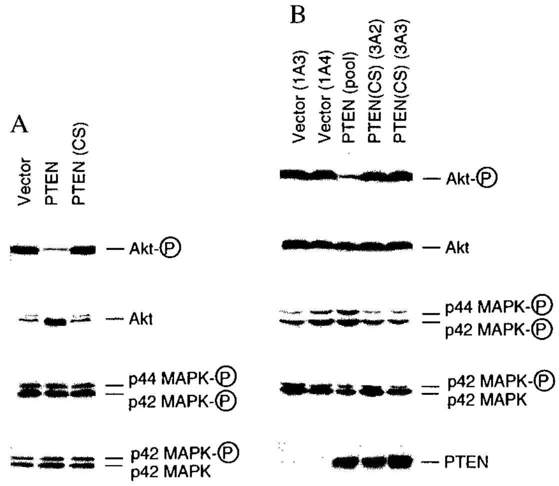

FIG. 4. Phosphorylation status of Akt and MAPKs. (*A*) Phosphorylation status of Akt and MAPKs in PTEN retroviruses-infected cells. U87-EcoR cells were infected with retroviruses carrying empty vector, PTEN, or PTEN(CS) and harvested 48 hr later. Cell lysates (50 μg each) were analyzed by Western blot analysis with specific antibodies against phospho-Akt or phospho-MAPKs, respectively. As controls, duplicate filters were examined in parallel with anti-Akt or anti-p42 MAPK antibodies that recognize both phospho- and nonphosphoproteins. (*B*) Phosphorylation status of Akt and MAPKs in U87MG derivatives that stably express PTEN or PTEN(CS). U87MG cell clones that contain the empty vector (clones 1A3 and 1A4) and the catalytically-inactive PTEN mutant, PTEN(CS) (clones 3A2 and 3A3); or the pooled clones from PTEN-expressing U87MG derivatives, PTEN (pool), were analyzed by Western blot analysis by using antibodies specific for the phosphorylated forms for Akt or MAPKs, respectively. As control, duplicate filters were examined in parallel with regular anti-Akt or anti-p42 MAPK antibodies. The expression of PTEN or PTEN(CS) were detected by Western blot analysis using affinity-purified anti-PTEN antibody.

electrophoretic mobility to faster migrating species, which could be resolved under certain electrophoresis condition (Fig. 4*A*). In contrast, the active and phosphorylated forms of both p42 and p44 MAPKs were not detectably altered by PTEN expression, as examined by using an antibody that recognizes the Thr-202/Tyr-204 phosphorylated forms of p42 and p44 MAPKs (Fig. 4*A*). As control, the level of p42 MAPK was also not affected by PTEN expression (Fig. 4*A*).

We further examined the phosphorylation status of Akt and MAPKs in U87MG derived cell lines that either stably express PTEN, its catalytically inactive mutant PTEN(CS), or the vector control. Western blot analysis of these cell lysates revealed that cells stably expressing PTEN, but not cells stably expressing the PTEN(CS) mutant, had greatly reduced phosphorylation of Akt, as compared with the vector control cells (Fig. 4*B*). Consistent with the transient expression assays, the phosphorylated forms of MAPKs were not affected in cell lines that stably express either PTEN or PTEN(CS) (Fig. 4*B*). PTEN and PTEN(CS) were expressed at the similar level in these cells (Fig. 4*B*), which were comparable to the endogenous PTEN protein levels in several cell lines such as DLD1 or MvLu (see Fig. 1*C*).

In addition, we have examined the tyrosine phosphorylation status of PI 3-kinase in these cells. The best-characterized PI 3-kinase, p85/p110 heterodimer, is known to be activated by tyrosine phosphorylation of p85 regulatory subunit, which leads to conformation change and thus activation of the p110 catalytic subunit (18). We had found that there was no change in the tyrosine phosphorylation level of the p85 subunit of PI 3-kinase in U87MG derivatives either transiently or stably expressing PTEN or PTEN(CS) (data not shown). Therefore, our studies indicate that PTEN expression leads to the selective inhibition of Akt signaling pathway and the inhibition of Akt phosphorylation is likely to occur downstream of PI 3-kinase.

**Inhibition of PI 3-Kinase by Pharmacological Reagents Leads to Up-Regulation of p27$^{KIP1}$ and Cell Cycle Arrest.** The inhibition of phosphorylation of Akt in PTEN-expressing cells suggests that PTEN may be involved in the PI 3-kinase signaling pathway. In addition, our studies suggest that PTEN expression causes the accumulation of p27$^{KIP1}$, raising the possibility that the PI 3-kinase signaling pathway may regulate the level of p27. To confirm that the PTEN-induced increase of p27 is caused by down-regulation of the PI 3-kinase signaling pathway, we have examined whether p27 accumulation can be mimicked by inhibition of PI 3-kinase pathway by using pharmacological agents. Log-phase growing U87-EcoR cells were treated with LY294002, an inhibitor that selectively inhibits PI 3-kinases. As shown in Fig. 5*A*, such treatment potently inhibited the phosphorylation of Akt and also led to a significant increase of p27 level. Similar to the LY294002 effect, treatment of cells with wortmannin, another inhibitor for PI 3-kinase, also led to reduction of Akt phosphorylation and up-regulation of p27 (data not shown). In contrast, treatment of cells with PD98059, a specific inhibitor of MEK (MAPK kinase), had very little effect on the p27$^{KIP1}$ protein level, whereas it readily inhibited the phosphorylation of p42 and p44 MAPKs (data not shown).

We further examined whether PTEN-mediated cell cycle arrest can be mimicked by the drug LY294002. As shown in Fig. 5*B*, treatment of U87-EcoR cells with LY294002 nearly abolished the S-phase population (>90% reduction of S phase), with a concomitant increase of $G_0/G_1$-phase population and a modest increase in the $G_2$/M-phase population. These studies further suggest that PTEN-mediated cell cycle arrest in U87 cells involves the inhibition of PI 3-kinase signaling pathway and one critical target for this signaling pathway is p27$^{KIP1}$.

We also examined the response in DLD1 cells toward LY294002 treatment. As discussed above, DLD1 appeared to be relatively resistant toward growth inhibition mediated by PTEN-expressing retroviruses (Fig. 1*A*). As shown in Fig. 5*A*, although LY294002 treatment of DLD1 cells readily inhibited the phosphorylation of Akt, however, it only modestly affected the level of p27$^{KIP1}$. It is worth pointing out that the steady-state level of phosphorylated Akt in DLD1 was severalfold lower than that in U87 cells when they were compared side by side (data not shown). In addition, LY294002 treatment led to a partial reduction of S phase population (≈40%) in DLD1 cells (Fig. 5*B*). These results showed that U87 and DLD1 cells exhibited different sensitivity toward inhibition of PI 3-kinase. One possible explanation for such different cell responses toward ectopic PTEN-expression or LY294002 treatment is that the relative contribution of PI 3-kinase signaling toward cell cycle progression is different in these two types of cells. Although PI 3-kinase signaling may be a rate-limiting step for p27$^{KIP1}$ down-regulation and cell cycle progression in U87 cells, additional pathways could be involved in DLD1 cells for these processes.

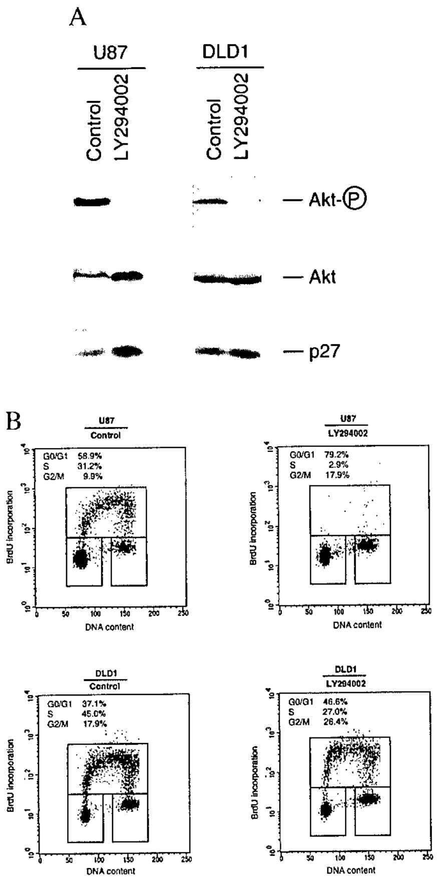

FIG. 5. Effect of LY294002 on Akt, p27$^{KIP1}$, and cell cycle profile. (*A*) Western blot analysis of drug-treated cells. Actively growing U87 cells (*Left*) or DLD1 cells (*Right*) were treated with either dimethyl sulfoxide (control) or LY294002 (20 μM) for 24 hr. Cell lysates (50 μg each) were examined by Western blot analysis with phosphorylation status specific or regular antibodies against Akt, respectively. A duplicated blot was also examined by Western blot analysis with anti-p27 antibody. (*B*) Fluorescence-activated cell sorting analysis of cell cycle profile of drug-treated cells. Actively growing U87 cells (*Upper*) or DLD1 cells (*Lower*) were treated with either dimethyl sulfoxide (control) or LY294002 (20 μM) for 24 hr. Cells were harvested and examined by fluorescence-activated cell sorting analysis for cell cycle distribution as described in the legend of Fig. 3*A*.

## DISCUSSION

Our studies have shown that expression of PTEN/MMAC1/TEP1 potently suppresses the growth and tumorigenicity of human glioblastoma U87MG cells *in vitro* and *in vivo*. This growth suppression activity is dependent on the intrinsic phosphatase activity of the enzyme. Our results are consistent with earlier minichromosome transfer experiments showing that introduction of human chromosome 10q into glioblastoma cells suppressed the ability of these tumor cells to grow in soft agar or form tumors in nude mice (20). Very recently, it has also been shown that adenovirus-mediated PTEN gene delivery, which expressed PTEN at very high level, also suppressed the growth of these cells in soft agar and in nude mice (21). Our retrovirus system has shown that PTEN can suppress tumorigenicity in U87MG cells at the expression level that is comparable to the endogenous PTEN protein level observed in many cell lines.

Our results strongly suggest that the growth suppression effect of PTEN in U87MG cells is mediated by its ability to block cell cycle progression at $G_1$ phase. The PTEN-induced cell cycle arrest is correlated with a significant accumulation of the cell cycle kinase inhibitor $p27^{KIP1}$. The importance of down-regulation of $p27^{KIP1}$ for $G_1$ cell cycle progression has been suggested. It has been reported that expression of a dominant-negative form of Ras, likely through inhibition of PI 3-kinase pathway, blocked the down-regulation of $p27^{KIP1}$ in NIH 3T3 cells at mid- or late-$G_1$ stage (16). In aortic smooth muscle cells, wortmannin treatment blocked cell cycle progression in late $G_1$, which was accompanied by failure to down-regulate $p27^{KIP1}$ (17). In U87MG glioblastoma cells, we have observed that transient expression of PTEN led to significant up-regulation of $p27^{KIP1}$ and $G_1$ cell cycle arrest. Both PTEN-induced $G_1$ cell cycle arrest and $p27^{KIP1}$ accumulation can be mimicked by treatment of cells with pharmacologic reagents that inhibited PI 3-kinases, suggesting that PTEN can negatively regulates the PI 3-kinase pathway. We have also shown that expression of PTEN leads to inhibition of Akt/PKB, a serine–threonine kinase activated by PI 3-kinase signaling pathway, and such inhibition appears to occur downstream of activation of PI 3-kinase. Very recently, it has been reported that the PTEN protein possesses a phosphatase activity toward phosphatidylinositol 3,4,5-trisphosphate (8). It is likely that in our system, the inhibition of Akt by PTEN expression is mediated by a reduction of the inositol phospholipid levels that are required for activation of this kinase.

PI 3-kinase activity has been shown to be required for $G_1$ to S progression in NIH 3T3 cells, as microinjection of neutralizing antibodies against PI 3-kinase inhibited growth factor induced DNA synthesis (22). Akt/PKB has been shown to be a critical downstream target of PI 3-kinase (19). Akt is an essential mediator for prevention of apoptosis in neuronal cells or lymphocytes after growth factor withdrawal (19). We have observed inhibition of Akt phosphorylation during PTEN-mediated cell cycle arrest. It is possible that Akt itself, or other molecules activated by PI 3-kinase signaling process, serves an essential function for $G_1$ cell cycle progression in U87MG glioblastoma cells. Such a process involves the down-regulation of CDK inhibitor $p27^{KIP1}$ during the $G_1$ progression.

In summary, our studies suggest that PTEN functions to down-regulate PI 3-kinase signaling pathway that normally plays an essential role in $G_1$ cell cycle progression. In the absence of PTEN tumor suppressor protein, the increased PI 3-kinase signaling may promote uncoordinated $G_1$ cell cycle progression, allowing cells to bypass the normal signaling processes regulated by growth factors and cell anchorage, leading to tumorigenesis.

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